## REMARKS

### I. Status of Claims

Claims 1-25 are pending in the application, and claims 6-14, 24 and 25 stand withdrawn pursuant to a restriction requirement. Claims 1-5 and 15-23 are under examination and stand rejected under 35 U.S.C. §112, first paragraph, 35 U.S.C. §112, second paragraph, 35 U.S.C., and 35 U.S.C. §102. The specific grounds for rejection, and applicants' response thereto, are set out in detail below.

### II. Sequence Listing Compliance

The office action objects to the specification as containing sequence disclosures without the use of sequence identifies. Given the number of amendments, applicants have prepared a substitute specification (attached hereto as Appendix A) to address the issue of sequence identifiers, as well as other informalities.

### III. Rejections Under 35 U.S.C. §112

#### A. First Paragraph (Enablement)

Claims 1-5 and 15-23 stand rejected as allegedly lacking an enabling disclosures. Applicants traverse.

The examiner sets forth a number of grounds for rejection, which applicants will attempt to address. First, there is a discussion of whether the specification supports the use of "any" polymorphism in UGT1A1 to assess risk of toxicity. OA at page 13. At present, applicants believe the examination is limited to the elected species of assessing the -3279 polymorphism (see page 2, paragraphs 1-2 of the action). Moreover, there is no indication in the action that the requirement for election of species has been withdrawn. Thus, the rejection is premature and should be held in abeyance until such time as the full scope of the claims is properly under

consideration. However, to the extent that the rejection addresses any issues that ***are*** properly under consideration, applicants note that the claims have now all been amended to recite the -3279 polymorphism.

Second, the examiner seems to argue that there is a problem correlating "any" toxicity, stating that there is insufficient information on the wide range of possible toxic effects, such as leucopenia, neotropenia, and diarrhea among others. OA at pages 6, 8 and 13. As written the claims do not call out correlation of specific genotypes with specific toxicities. The claims simply require that ***some*** toxicity risk be assessed, and thus the examiner seems to be imputing to the claims an outcome that is not required.

Third, the examiner also challenges how one can estimate toxicity risk when only a certain percentage of patients with the high risk genotypes actually do develop toxicity. OA at page 7. Again, the claims do not require ***identification*** of an individual who ***must*** develop toxicity, but rather, they merely recite evaluating risk, which encompasses determining a relative likelihood of developing toxicity, not any absolute predictability. Thus, again, the examiner seems to be imputing to the claims an outcome that is not required.

Fourth, the examiner challenges the ability of the specification to facilitate correlating a particular dose of irinotecan with any toxicity. OA at pages 7, 8 and 9. Again, applicants do not believe the claims require such, but only that the dosage could be adjusted (*e.g.*, up or down) depending on the relative risk of toxicity. The specification discloses that the amount of glucuronidation of irinotecan is different depending upon whether a T or a G is present at position -3279; similarly, the specification discloses that the amount of glucuronidation of irinotecan is different depending upon whether an A or a G is present at position -3156. See, for example, paragraphs [0198], [0223] and Tables 3 and 8 in the published application. The

specification provides a significant amount of support for the claims as pending. See, also, paragraphs [0191], [0192], [0193], [0219], Table 6 and Figure 5 of the published application for support regarding detecting the nucleotide sequence at positions -3279 or -3156 to determine a patient's risk for irinotecan toxicity.

Fifth, the examiner challenges generally the reliability of genetic analyses, citing various post-filing publications in support of that position. Applicants submit that the issues raised in Kroese *et al.* do not rise to the level of non-enablement of genetic testing. Rather, the article merely sets forth issues relating to such tests including steps that should be taken to ensure the best results possible. Indeed, the authors admonish against bypassing genetic testing, despite its imperfections. If one could not practice such inventions, there would be little need to make such a recommendation. Turning to the Lucentini article, it should be noted that this was ***not*** published in a peer-reviewed journal. To support Applicants' position, please see the attached abstract of Ando *et al.* (2005, *Invest. New Drugs*, 23(6):539-45), which discloses that using UGT1A1 in genetic testing is uniquely predictive and that, while standard precautions should be taken, genetic testing of UGT1A1 should continue. As such, it does not necessarily reflect the view of the skilled artisan in the field. Taken on their own, the few citations provided do not accurately reflect the state of the art in this field and they must only be considered in the context of the totality of the data published by researchers in this field. To place any serious consideration on these few citations on their own is an argument without merit. Applicants also provide abstracts of the following representative post-filing date references to demonstrate the enablement of the pending claims. For example, Kitagawa *et al.* (2005, *Pharmacogen. Genetics*, 15(1):35-41) confirmed a correlation between the -3279 polymorphism and irinotecan toxicity; Maruo et al. (2004, *Hum. Genet.*, 115(6):525-6) described the -3279 polymorphism as an

"enhancer" polymorphism; Girard et al. (2005, *Hepatology*, 42(2):448-57) demonstrated that the polymorphisms as -3279 and -3156 correlate with changes in the metabolism of a cancer drug other than irinotecan; and Ferraris et al. (2006, *Genet. Test.*, 10(2):121-5) demonstrated a correlation between the -3279 polymorphism and Gilbert's Syndrome and/or hyperbilirubinemia. As evidenced by the attached abstracts, Applicants' disclosure in the instant application regarding the importance of the -3279 polymorphism in irinotecan metabolism has been substantiated by others in the art since the instant application was filed.

Sixth, the examiner challenges the data provided in applicants' specification on the grounds that it does not establish that the -3279 polymorphism correlates with irinotecan toxicity or that it is correlated with the number of *UGT1A1* repeats. OA at pages 6, 7, 9 and 11. Applicants traverse. Certainly, considering any one parameter in isolation (SN-38 glucuorinidation, bilirubin, race) might appear to provide the basis for questioning the relevance of the -3279 polymorphism generally, it is submitted that the evidence of record ***as a whole*** does indicate a relationship between -3279 status and irinotecan toxicity.

The examiner has cited Innocenti *et al.* (2002) as not supporting the claimed invention. In point of fact, a T *versus* G at -3279 showed a small though non-significant decrease ("Concerning the -3279G>T variant, SN-38 glucuronidation was reduced in I/II pairs compared to I/I pairs among Caucasians, although without reaching statistical significance (2.06 ± 0.74 versus 2.53 ± 0.82 SN-38G/IS, respectively) (Wilcoxon rank sum test, P = 0.18"). Innocenti *et al.* (2005), also cited by the examiner, showed that for a -3279 T *versus* G comparison (1,1 *versus* 1,4) there appeared to be no difference in SN-38 glucuronidation; other parameters were not measured. Also, Ramirez *et al.* (2006) (attached) did not show an effect of -3279 mediating

UGT1A1 inducibility, and Kanai *et al.* (2005) (attached) did not show an effect on total bilirubin in Japanese neonates, but neither of these necessarily correlates with toxicity.

On the other hand, Kitagawa *et al.* (2005) (attached) showed an effect of the -3279G associated with increased irinotecan toxicity and reduced SN-38G/SN-38 ratios in Japanese patients, although the magnitude of the effect is small and data on the effect of *6 on AUC ratios was not reported. Sugatani *et al.* (2002) (previously submitted as C199) showed an effect of increased bilirubin of the G allele (case-control study in adults), and Ferraris *et al.* (2006) (attached) showed an effect of increased bilirubin of the G allele (case-control study in Italian pediatric patients). Finally, Ki *et al.* (2003) (attached) showed an association of -3279 status with bilirubin in adult Korean healthy subjects.

A review of the underlying data for these two groups of references is illustrative. For the citations made by the examiner – Innocenti *et al.* (2002), Ramirez *et al.* (2006) and Kanai *et al.* (2005) – the first two did not examine patient populations, and the third looked at 119 patients and 26 other subjects, all from a single ethnic group. To the contrary, the citations made by applicants – Kitigawa *et al.* (2005), Sugatani *et al.* (2002), Feraris *et al.* (2006) and Ki *et al.* (2003) – are all characterized by study of human populations (n = 119, 52, 53 and 324, respectively), and the significance of these finding was high, in one case a p value of $< 0.001$. Thus, if offered contradictory results, the skilled artisan would most certainly adopt the conclusions of applicants' citations given the greater vitality of the data cited therein.

Thus, according to the linkage disequilibrium differences among the UGT1A1 alleles in Caucasians vs. Asians, in the absence of the information on the *28 and the *6 alleles, -3279 could be informative of the UGT1A1 status overall. Due to the presence of the *6 allele (211G>A) in Asians, a T at -3279 might indicate normal UGT1A1 status in Caucasians but a

deficient UGT1A1 status in Asians, as shown in Innocenti *et al.* (2005). The conclusion, then, from all of the preceding data, is that -3279 is probably useful in predicting irinotecan toxicity in at least Asian populations,. This is sufficient to satisfy §112 enablement requirements, which only calls for a reasonable correlation with the claimed subject matter.

**B. First Paragraph (Written Description)**

Claims 1-5 and 15-23 are rejected as allegedly lacking an adequate written description. The basis of the rejection appears to be the allegation that neither "the specification nor the art teach 'any' polymorphism linkage with 'any' TA repeat in the UGT1A1 gene" or "any TA repeat in linkage disequilibirum with any polymorphism in the UGT1A1 gene that is associated with the risk of irinotecan toxicity." Applicants traverse.

At present, applicants believe the examination is limited to the elected species of assessing the -3279 polymorphism (see page 2, paragraphs 1-2 of the action). Moreover, there is no indication in the action that the requirement for election of species has been withdrawn. Thus, applicants believe the rejection is premature and should be held in abeyance until such time as the full scope of the claims is properly under consideration. However, to the extent that the rejection addresses any issues that ***are*** properly under consideration, applicants note that the claims have now all been amended to recite the -3279 polymorphism. The specification provides sufficient written description for the claims as amended. See, for example, paragraphs [0017], [0025], [0191], [0192], [0193], [0198], [0219], Table 6 and FIG. 5 of the published application. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

**C. Definiteness**

Claims 1-5 and 15-23 stand rejected as indefinite under the second paragraph of §112. The specific grounds for rejection are set out below:

**Claims 1-5 and 15-23**. The examiner argues that the independent claims fail to satisfy the preamble. Applicants have amended these claims to provide wherein clauses that are believed to satisfy the preamble.

**Claim 2**. Claim 2 is rejected for the language of "all or part of 5' flanking region ...." Applicants traverse, but in the interest of advancing the prosecution, the claims have been amended to remove the rejected language.

**Claim 3**. Claim 3 is rejected as indefinite. The claim has been canceled without prejudice or disclaimer to prosecution in a later filed case.

**Claim 21**. Claim 21 is rejected for use of the term "second agent." Applicants have deleted this terminology. The examiner also questions how provision of an additional agent can further limit a claim to assessing risk of toxicity. The answer to this question is quite simple – assessment of toxicity is a required aspect of the claim by virtue of its dependence on claim 1, while provision of an additional agent is only found in the dependent claim, thereby further limiting claim 1.

**Claim 23**. Claim 23 is rejected as allegedly lacking antecedent basis for the recitation of "the UGT1A1 activity." Applicants have amended the claim to address this issue. In addition, the relationship of UGT1A1 activity and irinotecan toxicity is said to be unclear. Again, applicants have deleted this language.

Reconsideration and withdrawal of the rejections is respectfully requested.

## IV. Rejections Under 35 U.S.C. §102

### A. Innocenti

Claims 1-5, 15, 17-19, 22 and 23 stand rejected as anticipated by Innocenti *et al.* (2002) under §102(a). Applicants traverse the rejection in light of the "Katz" declaration filed herewith, establishing that Innocenti *et al.* is not "by another" for the purposed of §102(a). As such, Innocenti *et al.* is not prior art against the instant application.

### B. Hasegawa

Claims 1-2, 4 and 16-20 stand rejected as anticipated by Hasegawa *et al.* (US2004/0058363) under §102(e). Applicants traverse, but in the interest of advancing the prosecution, claim 1 has been amended to incorporate the limitations of claim 5, which is not rejected. Therefore, the rejection is believed to be overcome.

## V. Conclusion

In light of the foregoing, applicants respectfully submit that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should the examiner have any questions regarding this response, a telephone call to the undersigned at (512) 536-3084 is invited.

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Original Article

# Neonatal hyperbilirubinemia and the bilirubin uridine diphosphate-glucuronosyltransferase gene: The common –3263T > G mutation of phenobarbital response enhancer module is not associated with the neonatal hyperbilirubinemia in Japanese

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**Abstract**

***Background***: Neonatal hyperbilirubinemia is frequent and severe in Japanese newborns. Previously, it has been reported that half of the Japanese neonates with severe hyperbilirubinemia carried the 211G > A (p.G71R) mutation of the bilirubin uridine diphosphate-glucuronosyltransferase (UGT1A1) gene causing Gilbert syndrome. Recently, it was reported that the –3263T > G mutation in the phenobarbital response enhancer module in *UGT1A1* was associated with the majority of cases of Gilbert syndrome. The gene frequency of the –3263T > G mutation was determined and the relation with neonatal hyperbilirubinemia in Japanese was studied.

***Methods***: *UGT1A1* in 119 neonates born at Yamagata University Hospital, Yamagata, Japan, and 26 subjects who had undergone phototherapy due to severe hyperbilirubinemia at four other hospitals were studied. The gene frequency of –3263T > G mutation in Japanese, Korean, Chinese and German healthy adult controls was also determined. Hyperbilirubinemia was assessed with a Jaundice Meter and *UGT1A1* was analyzed by sequence determination or restriction enzyme method.

***Results***: The gene frequency of the –3263T > G mutation was 0.26 in Japanese subjects and was similar to the prevalence in Korean, Chinese and German populations. However, there was no significant increase in the gene frequency of the mutation in the neonates who required phototherapy for hyperbilirubinemia compared to that in the neonates without severe hyperbilirubinemia. In addition, neonates with or without the mutation did not show a significant change in the level of bilirubin and the mutation also did not show a synergic effect with the 211G > A mutation on the level of bilirubin.

***Conclusion***: The –3263T > G mutation is not likely to be associated with the neonatal hyperbilirubinemia in Japanese.

**Key words** bilirubin uridine diphosphate-glucuronosyltransferase, Gilbert syndrome, neonatal hyperbilirubinemia, phenobarbital response enhancer.

Unconjugated hyperbilirubinemia in neonates is a physiological and very common phenomenon.[1] Bilirubin production is increased in the neonate because of the larger erythrocyte volume, shortened erythrocyte lifespan, heme and heme precursors degraded from the fetal extramedullary hematopoietic tissue, and, possibly, increased turnover of cytochromes.[2] In addition, the ability to conjugate bilirubin is extremely low in the neonate; the bilirubin uridine diphosphate-glucuronosyltransferase (UGT1A1) activity of neonates at term is about 1% of adult values.[3] Neonatal hyperbilirubinemia is also probably associated with other factors such as an immaturity of hepatic bilirubin uptake and intracellular bilirubin transport, and increased enterohepatic circulation of bilirubin. The imbalance between the

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production and the elimination of the bilirubin leads to various levels of hyperbilirubinemia in every neonate.

Recently, it was reported that the mutations of the *UGT1A1* gene causing Gilbert syndrome are associated with neonatal hyperbilirubinemia.[4-6] Neonatal hyperbilirubinemia in Japanese, Koreans and Chinese is well known to be more frequent and more severe than that in Caucasians and Negroes.[2] We found that the 211G > A mutation of the *UGT1A1* gene was associated with neonatal hyperbilirubinemia in Japanese, Koreans and Chinese.[5,6] However, this mutation was detected in only about 50% of Japanese neonates with severe hyperbilirubinemia and cannot explain the other half of Japanese neonates with severe hyperbilirubinemia. In other nations, the (TA)7TAA mutation (TA insertion in the TATA element of the *UGT1A1* gene) is associated with neonatal hyperbilirubinemia.[4] Recently, Sugatani *et al.*[7] reported that the –3263T > G mutation in the phenobarbital response enhancer module in the *UGT1A1* gene was associated with the majority of cases of Gilbert syndrome. In the present paper, we studied the prevalence of the –3263T > G mutation of the *UGT1A1* gene in various populations and the relationship between this mutation and neonatal hyperbilirubinemia in Japanese.

## Methods

### *Subjects and samples*

This study was approved by the Ethics Committee of the Yamagata University School of Medicine, Yamagata, Japan. With informed consent from the parents, we collected the umbilical cord blood of the neonates born at Yamagata University Hospital between 1999 and 2001 for genomic DNA extraction and hemoglobin separation. Neonates whose birthweights were < 2500 g and whose gestational age was < 36 weeks were excluded. We also excluded neonates who had factors which may have affected the level of serum bilirubin such as hemolytic anemia, neonatal asphyxia, maternal diabetes, congenital heart or intestine malformation, infections, drug administrations and parenteral fluid therapies. Thus, we finally studied 119 subjects with umbilical cord samples and three of them underwent phototherapy. In total, 26 subjects who had undergone phototherapy were also recruited from four hospitals in Yamagata prefecture between 1997 and 1998 and their peripheral blood was collected with their parents' consent for genomic DNA extraction. Genomic DNA samples of Japanese, Korean, Chinese and German controls collected from healthy adults were also used. Genomic DNA was extracted from the white blood cells by ordinary methods.

### *Hyperbilirubinemia assessment and criteria for phototherapy*

Hyperbilirubinemia of the neonates was assessed with a Jaundice Meter (model 102; Minolta, Osaka, Japan) once a day, at the same time, during hospitalization or at least during the first week of life. When the reading of the transcutaneous bilirubinometer (TCBR) reached the criterion for further evaluation, total serum bilirubin concentration was measured by the bilirubin oxidase method. Phototherapy was initiated if the measured bilirubin level exceeded the criterion as follows: 102.6 mmol/L at day 0, 171 mmol/L at day 1, 205.2 mmol/L at day 2, 239.4 mmol/L at day 3, 256.5 mmol/L at day 4, 273.6 mmol/L at day 5, 290.7 mmol/L at day 6 and 307.8 mmol/L at day 7 or greater.

### *UGT1A1 gene analysis*

The (TA)7TAA and –3263T > G mutations in the promoter region of the *UGT1A1* gene were analyzed as previously reported.[6,7] The 211G > A mutation was studied by *Esp*3I restriction analysis.

### *Statistical analysis*

The gestational age, birthweight and ratio of breast-fed to formula-fed were compared between the subjects who underwent phototherapy (PT group) and the subjects who did not require any treatment (non-PT group) using the unpaired *t*-test. Comparisons of the distributions between the two groups were analyzed using the $\chi^2$ test. Analysis of the TCBR among the three or six groups on each day was performed with the Kruskal–Wallis test, and TCBR of the two groups was compared using the Mann–Whitney *U*-test.

## Results

### *Comparison of the frequency of the –3263T > G mutation among Japanese, Korean, Chinese and German populations*

To study the distribution of the –3263T > G mutation, we compared the gene frequencies among Koreans, Chinese and German populations. The gene frequency in Japanese was 0.26 and was similar to those in other populations (Table 1).

### *Mutation analysis of the promoter and coding regions of the UGT1A1 gene in Japanese neonates who underwent phototherapy*

A total of 119 subjects with umbilical cord blood samples and 26 subjects who had undergone phototherapy were recruited for this study. A total of 29 subjects underwent

**Table 1** Allele frequency of –3263T > G mutation of the *UGT1A1* gene in various populations

| Ethnic background | Genotype distribution | | | | | |
|---|---|---|---|---|---|---|
| | *n* | Wild | Hetero | Homo | Allele frequency | Ethnic background |
| Japanese | 157 | 87 | 58 | 12 | 0.26 | NS |
| Korean | 55 | 24 | 26 | 5 | 0.33 | NS |
| Chinese | 50 | 26 | 18 | 6 | 0.30 | NS |
| German | 57 | 24 | 26 | 7 | 0.35 | NS |

Hetero, heterozygous; Homo, homozygous; NS, not significant.

**Table 2** Distribution of genotypes and allele frequencies of –3263T > G (TA)7TAA and 211G > A mutations of the *UGT1A1* gene in the phototherapy and non-phototherapy groups

| Mutation | PT group | | | | | non-PT group | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Genotype distribution | | | | | Genotype distribution | | | | |
| | *n* | Wild | Hetero | Homo | Allele frequency | *n* | Wild | Hetero | Homo | Allele frequency |
| 3263T > G | 29 | 19 | 9 | 1 | 0.19 | 116 | 65 | 43 | 8 | 0.25 |
| (TA)7TAA | 29 | 29 | 0 | 0 | 0 | 116 | 96 | 20 | 0 | 0.09* |
| 211G > A | 29 | 14 | 14 | 1 | 0.28 | 116 | 80 | 31 | 5 | 0.18 |

*$P < 0.05$ versus PT group.

Hetero, heterozygous; Homo, homozygous; PT, subjects who received phototherapy; non-PT, subjects who did not receive phototherapy.

phototherapy (PT group) and another 116 subjects did not require any treatment (non-PT group). There were no significant differences between the PT and non-PT groups in birthweight, gestational age or the ratio of breast-fed to formula-fed subjects (data not shown). Analysis of allele frequencies in 119 subjects using umbilical cord blood samples showed evidence for linkage disequilibrium between the –3263T > G (TA)7TAA and 211G > A mutations.

To elucidate the effects of the –3263T > G (TA)7TAA and 211G > A mutations on the bilirubin metabolism, we compared the gene frequency of each mutation between the PT group and non-PT group. The frequency of the –3263T > G mutation in the PT group was 0.19 and was rather low compared to 0.25 in frequency of the non-PT group (Table 2). The (TA)7TAA mutation was not detected in the PT group. The 211G > A mutation was detected in 52% of the neonates in the PT group and was more frequent compared to 31% of the neonates in the non-PT group. However, there was no significant difference in the gene frequency between the two groups.

### *Relation between the genotypes of the –3263T > G mutation and transcutaneous bilirubinometer*

To confirm the effect of the –3263T > G mutation on bilirubin metabolism of neonates, we also analyzed the relation between the genotypes and TCBR on days 0–5 in the neonates after excluding the subjects with the 211G > A or (TA)7TAA mutations of the *UGT1A1* gene. There was no significant increase in TCBR in the neonates heterozygous or homozygous for the –3263T > G mutation compared to neonates with no mutation on days 0–5 (Fig. 1).

Then, we studied the combination effect with the 211G > A mutation on TCBR in the neonates when we excluded the subjects with the (TA)7TAA mutation. As shown in Fig. 2, the TCBR of the neonates carrying the 211G > A mutation with or without the –3263T > G mutation tended to be higher than that of the neonates not carrying the 211G > A mutation with or without the –3263T > G mutation on days 4 and 5. When the TCBR was compared in the neonates subdivided only by the genetic state of the 211G > A mutation, there was a significant increase in the TCBR on day 5 of the neonates with the 211G > A mutation compared to that of the neonates without the 211G > A mutation (data not shown). However, we could find no significant difference in TCBR between the neonates carrying the 211G > A and –3263T > G combined mutations and the neonates carrying the 211G > A single mutation.

Few subjects carried the (TA)7TAA mutation, thus, we could not investigate the synergic effects of –3263T > G and (TA)7TAA mutations.

## Discussion

Neonatal hyperbilirubinemia is a physiological phenomenon, but its severerity is also affected by genetic factors.[1] We reported that the 211G > A mutation of the *UGT1A1* gene

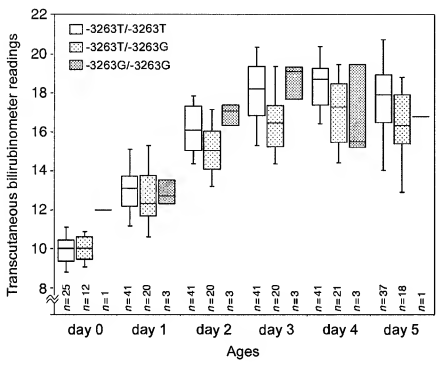


**Fig. 1** Transcutaneous bilirubinometer reading from days 0–5 in the wild, heterozygotes and homozygotes for the –3263T > G mutation. Boxes show median, 25% and 75%, and bars are 10% and 90% for each group. No significant difference was seen with the Kruskal–Wallis test on each day. '*n*' represents number of subjects tested.

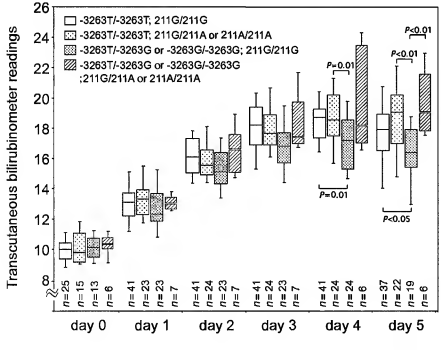


**Fig. 2** Transcutaneous bilirubinometer reading from days 0–5 in the four groups subdivided by the combination of –3263T > G and 211G > A mutations. Boxes show median, 25% and 75%, and bars are 10% and 90% for each group. Significant differences were seen with the Kruskal–Wallis test among the four subgroups on days 4 and 5. *, significant differences ($P < 0.05$) between two subgroups with Mann–Whitney *U*-test; $P = 0.01$, $P = 0.05$, *P*-value in Mann–Whitney *U*-test; '*n*' represents number of subjects tested.

was associated with the neonatal hyperbilirubinemia in Japanese, Koreans and Chinese.[5,6] However, half of the Japanese neonates with severe hyperbilirubinemia did not carry the 211G > A mutation. Phenobarbital administration has been known to increase expression of the *UGT1A1* gene in the liver. Sugatani *et al.*[8] recently identified a 290-bp enhancer sequence (–3483/–3194) of the *UGT1A1* gene, the phenobarbital response enhancer module. They also reported that the –3263T > G mutation in this module of the *UGT1A1* gene was associated with the majority of cases of Gilbert

syndrome.[7] They also found a synergic effect with the (TA)7TAA or 211G > A mutations on the level of plasma bilirubin. We confirmed that this mutation is also common among Japanese, Korean, Chinese and German populations and found linkage disequilibrium between the −3263T > G (TA)7TAA and 211G > A mutations. We could not find a statistically significant increase in the gene frequency of −3263T > G in the PT group compared to the frequency in the non-PT group. In addition, neonates with or without the −3263T > G mutation did not show a significant change in the level of bilirubin on days 0–5 and the mutation also did not show a synergic effect with the 211G > A mutation on the level of bilirubin. In contrast, there was a significant increase in the TCBR of neonates carrying the 211G > A mutation on day 5. The 211G > A mutation was significantly associated with neonatal hyperbilirubinemia in Japanese, as described in our previous papers.[5,6] Our data suggested that the −3263T > G mutation in the phenobarbital response enhancer module is not associated with neonatal hyperbilirubinemia in Japanese. The UGT1A1 activity in the neonates at birth is 0.1–1% of that in adults and increases exponentially to the level of adults by 14 weeks of age.[3] Japanese neonates with hyperbilirubinemia may have a defect in the induction system in the development of UGT1A1.

Hyperbilirubinemia is caused by the imbalance between the production and the elimination of the bilirubin. An increase in the bilirubin production had been speculated in Japanese neonates based on the finding that Japanese neonates with hypebilirubinemia produced more carbon monoxide (CO), probably via heme oxygenase reaction, compared with Japanese neonates without hypebilirubinemia.[8–12] Recently, we studied two notable factors that may be associated with bilirubin production in the neonate, the gene of heme oxygenase-1, a rate-limiting enzyme in heme metabolism, and the composition of fetal hemoglobin, a probable major source of bilirubin in neonates.[13] However, we could not find any characteristic change in those factors associated with neonatal hyperbilirubinemia in Japanese. Kaplan *et al.*[14] examined blood carboxyhemoglobin corrected for inspired CO (COHbc) and serum conjugated bilirubin fractions in neonates. They found that the neonates carrying the (TA)7TAA mutation of the *UGT1A1* gene in a homozygous state showed not only decreased serum conjugated bilirubin fractions, but also increased COHbc. The data of Kaplan *et al.*[14] strongly suggested that UGT1A1 promoter polymorphism (TA)7TAA mutation influences serum total bilirubin values by increasing heme catabolism as well as decreasing bilirubin conjugation. Considering that heme oxygenase is a stress-induced protein, we can speculate that neonates carrying the (TA)7TAA or 211G > A mutation of the *UGT1A1* gene decrease UGT1A1 activity, which may directly or indirectly induce the heme oxygenase and result in an increase in CO production.

The genetic or environmental factors involved in the steps between the production and the elimination of the bilirubin should be studied to elucidate the pathophysiology of neonatal hyperbilirubinemia.

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men classified as unlikely to be, uncertain to be, or likely to be vitamin $B_{12}$ deficient (Table 1A). HoloTC was significantly ($P$ <0.01) lower in the group of vegan men unlikely to have vitamin $B_{12}$ deficiency compared with omnivores, whereas this was not the case for B12 ($P$ = 0.30; Table 1A).

Finally we examined whether holoTC might replace combined testing with B12, MMA, and tHcy. In this analysis, individuals were classified as likely to have vitamin $B_{12}$ deficiency if at least two of three tests were outside the cutoff limits (B12 <120 ng/L, MMA >0.75 μmol/L, tHcy >15 μmol/L; n = 56) or not (n = 139; all others). The diagnostic accuracy of holoTC was then assessed by ROC curve analysis (Fig. 1B). The areas under the ROC curves were 0.91 (95% CI, 0.87–0.95) for all individuals, 0.88 (95% CI, 0.82–0.95) for vegan men recruited from the EPIC study, and 0.92 (95% CI, 0.85–0.99) for the men recruited from the London Vegan Society.

We also compared holoTC and the other three markers in omnivores and in vegan men classified as unlikely to be, uncertain to be, or likely to be vitamin $B_{12}$ deficient based on test results for B12, MMA, and tHcy (Table 1B). HoloTC was significantly lower ($P$ = 0.017) in the vegan men who were unlikely to be vitamin $B_{12}$ deficient compared with the omnivores, whereas no significant difference was observed for the other three measures: B12 ($P$ = 0.49), MMA ($P$ = 0.37), and tHcy ($P$ = 0.47).

Each of the laboratory tests used for diagnosis of vitamin $B_{12}$ deficiency has its weaknesses. The metabolites MMA and tHcy are influenced by kidney function (*10*, *11*), and tHcy also depends on the folate status of the patient. B12 is influenced by the concentration of the binding proteins of vitamin $B_{12}$ and is increased in patients with myeloproliferative diseases (*12*).

Our data are consistent with an early decrease of holoTC in vitamin $B_{12}$ deficiency in vegans and further suggest that holoTC might replace combined testing with B12, MMA, and tHcy in this population group. If holoTC is used as the primary screening test, we suggest no further testing for patients with holoTC >50 pmol/L (unlikely to suffer from vitamin $B_{12}$ deficiency) and holoTC <25 pmol/L (likely to suffer from vitamin $B_{12}$ deficiency). For patients with holoTC between 25 and 50 pmol/L, we would suggest further testing with one of the metabolic markers.

Further studies are needed to evaluate the validity of holoTC in other patient groups, especially those with conditions likely to influence markers of vitamin $B_{12}$ deficiency. Measurement of holoTC may be of particular value in identifying vitamin $B_{12}$ deficiency in patients with kidney malfunction, in whom the metabolites may show falsely increased values (*10*, *11*), and in patients with myeloproliferative diseases, in whom the concentration of B12 may be falsely increased (*12*). We conclude that holoTC is a promising indicator of vitamin $B_{12}$ deficiency.

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## Haplotype Structure of the UDP-Glucuronosyltransferase 1A1 (*UGT1A1*) Gene and Its Relationship to Serum Total Bilirubin Concentration in a Male Korean Population

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UDP-glucuronosyltransferase 1A1 (UGT1A1) is the key enzyme for bilirubin conjugation. Defects in this enzyme can cause a nonhemolytic unconjugated hyperbilirubinemia, such as Crigler–Najjar syndrome type 1 (CN1)

and 2 (CN2) and Gilbert syndrome (GS). In 1991, the cDNA of the human *UGT1A1* gene was cloned, and this led to the identification of genetic defects in patients with CN1, CN2, and GS *(1–3)*. It was shown that homozygous or compound heterozygous mutations of the *UGT1A1* gene can lead to these inheritable unconjugated hyperbilirubinemias, and >30 variants have been identified *(4, 5)*.

In GS, a TATAA box variant [$A(TA)_6TAA$>$A(TA)_7TAA$] in the promoter region of the *UGT1A1* gene has been reported in Caucasian populations, and several polymorphisms in the coding region, including 211G>A (G71R), have been reported to have similar associations with GS in Japanese populations *(6–8)*. Recently, Sugatani et al. *(9)* identified a T-to-G substitution in the phenobarbital-responsive enhancer module 3279 bp upstream from the *UGT1A1* gene. They suggested that the −3279T>G polymorphism could be another risk factor for the development of mild hyperbilirubinemia

Presumably, different combinations of the polymorphisms (haplotypes) in the *UGT1A1* gene associated with GS or mild hyperbilirubinemia might produce a variety of serum total bilirubin (T-Bil) concentrations. Because these polymorphisms in the *UGT1A1* gene lie in a relatively small region (Fig. 1A), a certain extent of linkage disequilibrium (LD) among these polymorphisms is expected. Therefore, haplotype analysis is more reasonable than association analysis using any single polymorphism to reveal the genetic background of an increased serum T-Bil concentration. We analyzed the haplotype structure of the *UGT1A1* gene and investigated its relationship to the serum T-Bil concentration in healthy Korean males.

The study participants were 324 healthy Korean males [mean (SD) age, 49.8 (5.4) years] randomly selected from the registry for routine health checks at Samsung Medical Center in Seoul, Korea. We restricted the study participants to males because the prevalence of unconjugated hyperbilirubinemia is higher in men than in women because of the lower rate of daily bilirubin production or other unidentified factors in females *(10, 11)* and thus the genetic influence on the bilirubin concentration might be more evident in men. None had a history of hepatic or hematologic disorders such as anemia, excessive alcohol intake, or chronic use of medications or narcotics. Informed consent was obtained from all participants, and the Institutional Review Board of the Samsung Medical Center approved the study protocol. Serum samples were collected from each participant in the morning after overnight fasting. The serum T-Bil concentration was measured by the diazo method (Daiichi Pure Chemicals) with the Hitachi 747 system (Hitachi) at least twice with a >1-month interval to provide a mean value for each participant. The mean values were used in all statistical analyses.

Two promoter regions containing the −3279T>G polymorphism and the TATAA box, and a part of exon 1 containing the 211G>A polymorphism of the *UGT1A1* gene were amplified and sequenced with use of appropriate primers, as given in the Data Supplement that accompanies the online version of this Technical Brief (available at http://www.clinchem.org/content/vol49/issue12/). The differences in the serum T-Bil concentration among the subgroups according to the *UGT1A1* alleles or haplotypes were tested by ANOVA. The influence of smoking status, polymorphisms, and haplotypes of the *UGT1A1* gene on serum T-Bil was also estimated by the GLM procedure incorporated in the SAS System for Windows (SAS Institute Inc.). The Hardy–Weinberg equilibrium was tested with Arlequin software *(12)*. Multisite haplotypes and their frequencies were estimated with use of SAS Genetics software (SAS Institute Inc.), and the assignment of haplotypes to each individual was performed with the PHASE program *(13)*. Pairwise LD was estimated as $D$, $D'$, and $r^2$ *(14)*. These LD coefficients were calculated with use of the SAS Genetics software.

The mean (SD) serum T-Bil concentration in the total group of participants was 16.3 (5.9) μmol/L [0.95 (0.34) mg/dL]. When we investigated the association of smoking with serum T-Bil concentrations, we observed a significant difference among groups by smoking status ($P$ = 0.0058, ANOVA). Current smokers had lower serum T-Bil concentrations [14.3 (5.3) μmol/L; n = 71] compared with

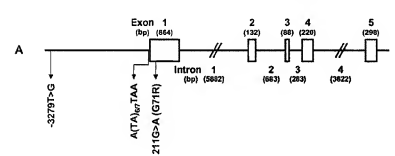


Fig. 1. Schematic presentation of the *UGT1A1* gene (*A*), and LD coefficients (*B*).

(*A*), exact sizes of exons (*open boxes*) and introns are based on the Ensembl transcript ENST00000330771 (http://www.ensembl.org). Locations of three common polymorphisms are indicated by *arrows*. (*B*), Lewontin's coefficient ($D'$) and correlation coefficient ($r^2$) between each pair of the three polymorphisms.

| Locus 1 | Locus 2 | $\|D'\|$ | $r^2$ |
|---|---|---|---|
| -3279T>G | $A(TA)_{6/7}TAA$ | 1 | 0.3999 |
| -3279T>G | 211G>A (G71R) | 1 | 0.0990 |
| $A(TA)_{6/7}TAA$ | 211G>A (G71R) | 1 | 0.0393 |

never smokers [17.9 (6.6) μmol/L; n = 45], and former smokers had serum T-Bil concentrations [16.8 (5.8) μmol/L; n = 155] between those of current smokers and never smokers. Smoking status was estimated to explain ~3.8% of the variation in serum T-Bil concentrations.

The genotype frequencies of all three polymorphisms were in Hardy–Weinberg equilibrium, and the frequencies of the −3279G, $(TA)_7$, and 211A alleles were calculated as 0.267, 0.127, and 0.213, respectively. As shown in the table in the online Data Supplement, the mean serum T-Bil concentrations in homozygous carriers of the −3279G, $(TA)_7$, or 211A allele were significantly higher than those in heterozygous carriers or homozygous carriers of wild-type alleles ($P$ <0.0001). Among the three polymorphisms, homozygous carriers of $(TA)_7$ showed the highest mean serum T-Bil concentration [33.2 (4.1) μmol/L], followed by homozygous carriers of 211A [24.2 (8.3) μmol/L] and −3279G [23.7 (6.2) μmol/L]. The variabilities in serum T-Bil explained by the −3279T>G, $(TA)_{6/7}$, and 211C>A polymorphisms were ~15.1% ($P$ <0.0001), 28.1% ($P$ <0.0001), and 12.9% ($P$ <0.0001), respectively.

A likelihood ratio test using Arlequin software *(12)* detected significant pairwise LD with $D'$ values of 1.0 between all pairs of the three polymorphisms ($P$ <0.0001; Fig. 1B). The highest correlation was observed between −3279T>G and $(TA)_{6/7}$ polymorphisms ($r^2$ = 0.3999; Fig. 1B). Multisite haplotype inference revealed that the participants had four of eight possible haplotypes: 3279T-$(TA)_7$-211G (ht1), 3279T-$(TA)_7$-211A (ht2), 3279G-$(TA)_7$-211G (ht3), and 3279G-$(TA)_7$-211G (ht4). ht1 was the most common with an estimated frequency of 0.5201 [95% confidence interval (CI), 0.4816–0.5586], followed by ht2 (0.2129; 95% CI, 0.1814–0.2445), ht3 (0.1404; 95% CI, 0.1137–0.1672), and ht4 (0.1265; 95% CI, 0.1009–0.1521).

When the participants were stratified into 10 groups according to their *UGT1A1* haplotypes, we observed significant differences in the mean serum T-Bil concentration among the groups ($P$ = 0.0001; Table 1). The lowest concentration was observed in group A [ht1/ht1; 13.1 (3.6) μmol/L], whereas the highest concentration was observed in group J [ht4/ht4; 33.2 (4.1) μmol/L]. Homozygous as well as compound heterozygous carriers of the variant haplotypes (ht2, ht3, or ht4) had increased serum T-Bil concentrations compared with homozygous or heterozygous carriers of the wild haplotype (ht1). Approximately 61.4% and 53.5% of the variability in serum T-Bil concentrations could be explained by the *UGT1A1* haplotype with or without consideration of smoking status, respectively.

Multisite haplotype inference revealed that our study population had only four of eight possible haplotypes. The four missing haplotypes, 3279T-$(TA)_7$-211G, 3279T-$(TA)_7$-211A, 3279G-$(TA)_6$-211A, and 3279G-$(TA)_7$-211A, suggest that −3279T plus $(TA)_7$ and $(TA)_7$ plus 211A alleles never exist on the same chromosome, at least in a Korean population. From the above results, we also suggest that a transition of G to A at nucleotide 211 in exon 1 of the *UGT1A1* gene would form the ht2 haplotype, whereas a transversion of T to G at nucleotide −3279 in the promoter region of the *UGT1A1* gene would form the ht3 haplotype. In addition, ht4 could be the result of an introduction of an extra (TA) repeat to the ht3.

**Table 1. Haplotype groups and T-Bil concentrations based on different combinations of three polymorphisms of the *UGT1A1* gene in 324 healthy Korean males.**

| Group | Haplotype[a] | n (%) | Mean (SD) T-Bil[b] μmol/L | mg/dL |
|---|---|---|---|---|
| A | ht1/ht1 | 90 (27.8) | 13.1 (3.6) | 0.76 (0.21) |
| B | ht1/ht2 | 66 (20.4) | 15.5 (3.1) | 0.91 (0.18) |
| C | ht2/ht2 | 18 (5.6) | 23.7 (6.2) | 1.38 (0.36) |
| D | ht1/ht3 | 54 (16.7) | 13.5 (2.8) | 0.79 (0.16) |
| E | ht2/ht3 | 17 (5.2) | 16.9 (3.4) | 0.99 (0.20) |
| F | ht1/ht4 | 37 (11.4) | 16.3 (4.5) | 0.95 (0.26) |
| G | ht2/ht4 | 19 (5.9) | 24.6 (5.1) | 1.44 (0.30) |
| H | ht3/ht3 | 4 (1.2) | 18.8 (1.7) | 1.10 (0.10) |
| I | ht3/ht4 | 12 (3.7) | 20.7 (7.3) | 1.21 (0.43) |
| J | ht4/ht4 | 7 (2.2) | 33.2 (4.1) | 1.94 (0.24) |

[a] ht1, 3279T-$(TA)_6$-211G; ht2, 3279T-$(TA)_6$-211A; ht3, 3279G-$(TA)_6$-211G; ht4, 3279G-$(TA)_7$-211G.

[b] There were significant differences in concentrations among the genotype groups ($P$ <0.0001, ANOVA).

Disclosure of the molecular genetic basis of reduced expression of UGT1A1 is important not only for understanding the molecular pathophysiology of increased serum T-Bil concentrations in GS but also for predicting severe toxicity by irinotecan in cancer patients *(15, 16)*. In addition, increased serum T-Bil concentrations have been associated with a low risk of coronary artery disease *(17, 18)*. To reveal the genetic background of an increased serum T-Bil concentration, haplotype analysis is more reasonable than association analysis using any single polymorphism because of tight LD among polymorphisms in the *UGT1A1* gene.

In summary, we demonstrated that there is complete LD among three common polymorphisms of the *UGT1A1* gene in a male Korean population. In addition, we could unequivocally construct haplotypes for the *UGT1A1* gene, which were revealed to be significantly associated with the serum T-Bil concentration. However, there are two major limitations in the present study. One is that the study population comprised only Korean males and thus the results cannot be freely applied to other populations, and the other is that only three common polymorphisms were included in the analysis. Nevertheless, to the best of our knowledge, this is one of the first studies to analyze the haplotype structure of the *UGT1A1* gene including both promoter and coding regions and to assess the haplotype–phenotype correlation between the *UGT1A1* gene and the serum T-Bil concentration.

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## Recombinant Human Intrinsic Factor Expressed in Plants Is Suitable for Use in Measurement of Vitamin $B_{12}$

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Competitive binding approaches with use of specific binding proteins are the most commonly used methods to measure vitamin $B_{12}$ in laboratory medicine. Various binding proteins have been used in these methods, including intrinsic factor (IF), pooled human or chicken serum, transcobalamin (TC), and saliva (*1*, *2*). The most widely used of these are non-human IF preparations, usually obtained from hog gastric mucosa. Numerous problems have been reported with their application, however, including difficulty of purification, instability on storage, and variation in the binding capacity connected with changes in serum protein and vitamin $B_{12}$ concentrations (*1*, *2*). If IF is not highly purified, it may contain haptocorrins (also called R proteins), which bind not only vitamin $B_{12}$ but also related metabolically inactive compounds that may be present in the sample, thereby causing artificially increased vitamin $B_{12}$ results (*1*, *3*).

To circumvent the problems associated with nonhuman IF, we recently expressed human IF in plants and obtained a product free of endogenous vitamin $B_{12}$ and contaminating vitamin $B_{12}$-binding proteins (*4*). In the current study, we examined the feasibility of using this recombinant human IF for measurement of vitamin $B_{12}$ bound to TC.

Human IF was expressed in the recombinant plant *Arabidopsis thaliana* and purified as described previously (*4*). As a first step, recombinant human IF was coupled to magnetic beads (Dynabeads, M-280 Tosylactivated; DYNAL) according to the protocol for ligand-binding applications as recommended by the manufacturer. The beads from 1 mL of the standard suspension were washed three times in 0.2 mol/L phosphate-buffered saline, pH 7.4. The washed beads were mixed with 1 mL of recombinant human IF (0.5 g/L) and incubated for 24 h at 37 °C with gentle and continuous agitation. The beads were precipitated, 1 mol/L Tris (pH 7.5) was added to the pellet, and the incubation was then continued for 4 h. The treated beads were then washed five times with 2 mL of 0.05 mol/L Tris (pH 7.5)–0.5 mol/L NaCl and suspended in this buffer.

Recombinant human IF coupled to magnetic beads was used for measurement of vitamin $B_{12}$ in the last step of the Axis-Shield Holo-TC assay in place of the binding protein supplied with the assay. This step measures the vitamin $B_{12}$ bound to TC trapped by insolubilized antibodies (*5*). The Axis-Shield Holo-TC assay was used to demonstrate

# Genetic polymorphism in the phenobarbital-responsive enhancer module of the *UDP-glucuronosyltransferase 1A1* gene and irinotecan toxicity

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**Genetic polymorphism of the *UDP-glucuronosyltransferase (UGT) 1A1* gene is associated with the decreased glucuronidation activity of an active metabolite of irinotecan, SN-38, and *UGT1A1*28* has been shown as a predictive factor for irinotecan toxicity. The phenobarbital-responsive enhancer module (PBREM) of the *UGT1A1* promoter region has been reportedly associated with the transcriptional activity of the gene. We investigated whether the polymorphism of PBREM (T-3279G) would affect interpatient variations in sensitivity to irinotecan toxicity. The study population comprised 119 cancer patients who had received irinotecan. We reviewed their clinical records, including patient characteristics, and observed their toxicity levels following irinotecan infusion. Genotyping was performed by sequencing analyses. Logistic regression analyses were performed to assess the relationship between genotypes and irinotecan toxicity. We identified the homozygotes of the reference allele for T-3279G in 68 patients, the heterozygotes in 37, and the homozygotes for the variant in 14. Logistic regression analysis indicated a significant association between the homozygotes for T-3279G and the severe toxicity (odds ratio 5.80; 95% confidence interval 1.67–20.1). However, multivariate analysis, including the data of *UGT1A1*28* polymorphism, revealed a diminution of the association due to a highly significant linkage disequilibrium between these polymorphisms. Our results suggest that a highly significant linkage disequilibrium exists between T-3279G and *UGT1A1*28* polymorphisms, and that the variants of T-3279G and *UGT1A1*28* cooperatively decrease transcriptional activity of the *UGT1A1* promoter. The determination of T-3279G and *UGT1A1*28* genotypes might be clinically useful in predicting severe irinotecan toxicity in cancer patients. *Pharmacogenetics and Genomics* 15:35–41**


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## Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11) is a camptothecin analogue with significant antitumour activity that inhibits topoisomerase I, and is now widely used in the treatment of patients with colorectal or lung cancer [1,2]. Irinotecan is hydrolysed by carboxylesterase to form SN-38 (7-ethyl-10-hydroxycamptothecin), which has a 100- to 1000-fold higher antitumour activity compared to the parent drug [3]. SN-38 is further conjugated by UDP-glucuronosyltransferase (UGT) 1A1 in the liver to yield SN-38 glucuronide, which has a 100-fold lower antitumour activity compared to SN-38 [4]. The SN-38 glucuronide is excreted in the small intestine via bile where bacterial glucuronidase resolves the glucuronide into the former SN-38 and glucuronic acid [3]. Some of the SN-38 is reabsorbed from the intestine into the body, resulting in the enterohepatic circulation of SN-38.

Irinotecan often causes an unpredictably severe, and occasionally fatal, toxicity of leukopenia or diarrhoea. Myelosuppression has been related to the plasma SN-38 area under the curve [5,6], although the inter-subject variations were significant and the mechanisms remain under investigation [7]. The direct effect of excreted SN-38, as modulated by its intraluminal formation from SN-38G by β-glucuronidase on the intestinal mucosa, has been proposed as a mechanism for irinotecan-related diarrhoea [8]. Increased plasma SN-38 is thought to induce the severe toxicities of myelosuppression or diarrhoea. Genetic polymorphisms of the *UGT* gene associated with reduced transcriptional and functional activity have been reported previously [3,9]. In patients

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with *UGT1A1*28* variants, which is a 2-bp insertion (TA) in the TATA box of the promoter region, the SN-38/SN-38 glucuronide AUC ratios in patients were significantly higher compared to the reference allele [10,11]. We have previously indicated that *UGT1A1*28* might explain the inter-individual differences in irinotecan toxicity [12].

Sugatani *et al.* [13] identified the region of the 290-bp phenobarbital-responsive enhancer module (PBREM) of the *UGT1A1* gene-promoter area, approximately 3 kb upstream of the TATA box [13]. PBREM contains three putative nuclear receptor motifs (NR4, gtNR1 and NR3), and is activated by the nuclear orphan receptor, human constitutive androstane receptor (hCAR). gtNR1 was shown to be the binding site of hCAR, whereas NR3 and NR4 also appeared to be required to confer full enhancer activity. Phenobarbital treatment has been used to reduce hyperbilirubinemia in an infant with Crigler–Najjar type II disease [14]. These findings suggest that PBREM may enhance the transcriptional activity of the *UGT1A1* promoter [13].

A recent report revealed the genetic polymorphisms of PBREM in the *UGT1A1* gene promoter [15]. Using sequence analysis, it was found that 21 of 25 patients with hyperbilirubinemia were genotyped for the T-3279G variant (position from the first base of the *UGT1A1* start site in the *UGT1A* cluster sequence AF297093, which is the same position reported as T-3263G by Sugatani *et al.* [13]). The decreased transcriptional activity of the T-3279G variant was also shown by a luciferase-reporter assay *in vitro* [15]. Based on those findings, and in addition to those of *UGT1A1*28*, we evaluated the risk of genetic polymorphisms of T-3279G that may influence the glucuronidation of SN-38 on the occurrence of severe toxicity after irinotecan treatment.

## Patients and methods

### Study population characteristics

The study population comprised 119 cancer patients, including 117 patients previously enrolled in a protocol entitled 'Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis' [12], plus two additional patients (Table 1). We reviewed their clinical records, including patient characteristics (age, gender, primary disease, previous treatments, evidence of distant metastasis, Eastern Cooperative Oncology Group performance status and major complications), dosage and schedule of irinotecan administration and concurrent use of other drugs or radiotherapy, and observed toxicity following irinotecan infusion (Table 1). Because the dose-limiting toxicities of irinotecan are myelosuppression and diarrhoea [2], 'severe toxicity' was defined as leukopenia of grade 4 ($\leq 0.9 \times 10^9$/l) and/or diarrhoea of grade 3 or worse (grade 3, watery for 5 days or more; grade 4, haemorrhagic or dehydration), as classified in accordance with the Japan Society for Cancer Therapy criteria [16]. We then identified 27 patients who experienced severe toxicity and 92 patients who did not. All the patients provided their informed written consent for their peripheral blood to be used in the research. The study was approved by the Ethical Committee of the Nagoya University School of Medicine and those of the participating institutes.

### Genotyping

Genomic DNA was prepared from whole blood (100–200 μl) using the QIAamp Blood Kit (Qiagen GmbH,

**Table 1 Baseline characteristics of patients**

| | Leukopenia (grade 4) and/or diarrhoea (grade 3 or worse)[a] | | *P* |
|---|---|---|---|
| | Experienced ($n=27$) | Not experienced ($n=92$) | |
| Gender (men/women) | 14/13 | 66/26 | 0.058[b] |
| Median age (range, years) | 58 (38–76) | 61 (41–75) | >0.2[c] |
| Performance status | | | >0.2[b] |
| 0 | 8 (30%) | 31 (34%) | |
| 1 | 16 (59%) | 50 (54%) | |
| ≥ 2 | 3 (11%) | 11 (12%) | |
| Primary disease | | | >0.2[b] |
| Small cell lung | 5 (19%) | 17 (18%) | |
| Non-small cell lung | 16 (59%) | 49 (53%) | |
| Colorectal | 3 (11%) | 18 (20%) | |
| Other | 3 (11%) | 8 (9%) | |
| Distant metastases | 22 (81%) | 68 (74%) | >0.2[b] |
| Previous treatment | | | >0.2[b] |
| None | 13 (48%) | 35 (38%) | |
| Systemic chemotherapy | 12 (44%) | 48 (52%) | |
| Surgery | 8 (30%) | 34 (37%) | |
| Radiotherapy | 3 (11%) | 17 (18%) | |
| Complications | | | >0.2[b] |
| Diabetes | 3 (11%) | 9 (9%) | |
| Liver diseases | 3 (11%) | 6 (7%) | |

[a]Japan Society for Cancer Therapy criteria.
[b]Chi-square test.
[c]Mann–Whitney U test.

Hilden, Germany). The variant T-3279G alleles of DNA polymorphism in PBREM were genotyped using a previously described method [15]. Briefly, polymerase chain reaction (PCR) amplification was performed using forward primer, 5′-CACCTCCTCCTTATTCTCTT-3′ and reverse primer, 5′-CTCATTCCTCCTCTCTAGCC-3′. The amplification reaction mixture (50 μl) contained 100 ng DNA in 0.2 mmol/l each of deoxynucleoside triphosphate, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 1.6 μmol/l of each primer, and 1.3 U of *Taq* polymerase (Takara Shuzo, Otsu, Japan). The PCR conditions were: 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 40 s (GeneAmp PCR System 2700, Applied Biosystems, Foster City, California, USA). The variant T-3279G allele was distinguished from the most common allele by direct sequencing (−3559 to −3160) of 400 bp produced by PCR, which includes three potential nuclear receptor motifs designated as NR4, gtNR1 and NR3. Cycle sequencing was performed with a dye-terminator sequence reaction (ABI Prism DNA Sequencing Kit, Perkin-Elmer, Foster City, California, USA) using an ABI PRISM 310 Genetic Analyser.

*UGT1A1*28*, a two-extra-nucleotide insertion (TA) within the TATA box resulting in the sequence $(TA)_7TAA$ (−39 to −53, *UGT1A1*28*) was distinguished from the most common allele (*UGT1A1*1*) by direct sequencing (−147 to +106) of 253–255 bp produced by PCR [17,18]. PCR amplification was performed using forward primer, 5′-AAGTGAACTCCCTGCTACCTT-3′ and reverse primer, 5′-CCACTGGGATCAACAGTATCT-3′. The amplification reaction mixture (50 μl) contained 100 ng DNA in 0.2 mmol/l each of deoxynucleoside triphosphate, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 1.6 μmol/l of each primer, and 1.3 U of *Taq* polymerase (Takara Shuzo). The PCR conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 40 s (GeneAmp PCR System 2700, Applied Biosystems). Cycle sequencing was performed with a dye terminator sequence reaction (ABI Prism DNA Sequencing Kit, Perkin-Elmer) using an ABI PRISM 310 Genetic Analyser.

### Blood sampling and plasma concentration of irinotecan and its metabolites

Blood samples were obtained from patients who had received irinotecan-based chemotherapy. Patients were confirmed to have adequate bone marrow and organ function before the use of irinotecan. Blood was drawn at the first administration of irinotecan on day 1, and plasma samples were obtained 0, 15, 30 and 60 min, and 2, 4, 8 and 24 h, respectively, after the irinotecan infusion. Plasma concentrations of SN-38 and SN-38G were analysed at a Research Laboratory, Daiichi Pharmaceutical Co. Ltd, Tokyo, using a modified reverse-phase high-performance liquid chromatographic (HPLC) method [7]. Briefly, a 0.1-ml plasma sample was mixed with a 0.1-ml HCl (0.01 mol) and camptothecin as an internal standard. The 0.1 ml mixed solution was applied to an automatic cleanup apparatus (PROSPEKT, Spark, Holland) for solid-phase extraction using the $C_{18}$ cartridge column. HPLC conditions for analysis of SN-38 were: pump, Shimadzu LC-10 AD; column, TSK gel ODS-80TM (150 × 4.6 mm; Tosoh, Tokyo, Japan); mobile phase, a mixture of acetonitrile and water (1 : 2, v/v); fluorescence detector, an excitation of 380 nm and an emission of 556 nm; retention time, 5.0 min. The SN-38G level was subsequently analysed using a similar method: mobile phase, a mixture (55 : 45) of methanol and 0.1 mol phosphate buffer with 3 mmol sodium 1-heptanesulfonate (pH 4.0); fluorescence detector, an excitation of 370 nm and an emission of 430 nm; retention time, 2.7 min. The lower limit of quantification was 2 ng/ml for both SN-38 and SN-38G. $AUC_{0-t}$ (μM h) from the beginning of the infusion to the last sampling time was calculated by the linear trapezoidal rule, using a computer program, TopFit 2.0 (Gustav Fisher, Verlag, Germany). Ratios of the $AUC_{SN-38G}/AUC_{SN-38}$ (μM h/μM h) were determined.

### Statistical analysis

Chi-square or Mann–Whitney *U*-tests were used to assess the correlation or association between patient characteristics and toxicity, as well as the distributions of genotypes between the patients who experienced severe toxicity and those who did not. Simple logistic regression analysis was used to assess the relationship between genotypes and toxicity. The odds ratio (OR) with a 95% confidence interval (CI) was calculated to determine any association between the *UGT1A1* variants and severe irinotecan toxicity. Possible variables that appeared to be associated with severe toxicity were considered for inclusion in an unconditional multiple logistic regression analysis. The importance of the genetic polymorphism for an occurrence of severe toxicity was verified when controlling for the other variables. Analyses were performed using SAS statistical software version 6.12 (SAS Institute Inc., Cary, North Carolina, USA). A two-tailed $P < 0.05$ was considered statistically significant.

## Results

### Genotyping of PBREM

The genotypes of PBREM were determined in 119 cancer patients who were previously treated with irinotecan. The sequences included three potential nuclear receptor motifs that were designated NR4, gtNR1 and NR3 and analysed. The polymorphism of T-3279G was detected in the NR3 domain of PBREM. We found homozygotes for the reference allele (T/T) in 68 patients, heterozygotes (T/G) in 37, and homozygotes for the variant T-3279G allele (G/G) in 14. The allele frequency of the variant T-3279G was 0.273 in our Japanese cancer population. We could not detect any

polymorphisms either in the NR4 or the gtNR1 domain, which are binding sites for the human constitutive androstane receptor (hCAR) [13]. No variants of C-3440A and T-3401C could be found [19].

### PBREM genotype and irinotecan toxicity

We collected clinical information from 119 Japanese cancer patients (Table 1). Nine (8%) and 38 (32%) patients experienced leukopenia of grade 4 ($0.9 \times 10^9$/l) and grade 3 ($1.9–1.0 \times 10^9$/l), respectively. Diarrhoea was reported in three patients (3%) with grade 4 (hemorrhagic or dehydration) and 20 (17%) with grade 3 (watery for 5 days or more). Five of the nine patients with grade 4 leukopenia also had grade 3/4 diarrhoea, and 17 of the 23 patients with grade 3/4 diarrhoea encountered grade 3/4 leukopenia. Thus, we identified 27 patients who experienced severe toxicity and 92 patients who did not (Table 1).

Distributions of T-3279G were significantly different in those two patient groups ($P = 0.012$) (Table 2). Simple logistic regression analysis showed a significant association between the homozygotes for T-3279G and the occurrence of severe toxicity (OR 5.80; 95% CI 1.67–20.1). This finding suggests that the homozygotes for T-3279G might be a significant predictor of severe irinotecan toxicity.

### PBREM genotype and *UGT1A1*28* genotype

A recent report demonstrated the linkage disequilibrium between the PBREM and *UGT1A1*28* variants in Caucasians and African-Americans [19]. Therefore, we compared the T-3279G variant with *UGT1A1*28* (Tables 2 and 3). We found homozygotes of the reference allele for *UGT1A1*28* [$(TA)_6/(TA)_6$] in 95 patients, heterozygotes [$(TA)_6/(TA)_7$] in 17, and homozygotes for the variant [$(TA)_7/(TA)_7$] in seven. Twenty-three of the 119 patients had both T-3279G and *UGT1A1*28* variants (Table 3). Twenty-eight patients had the T-3279G allele without *UGT1A1*28* variants. Only one patient had a single variant for *UGT1A1*28*. These findings suggest that the position –3279 in PBREM preferentially takes G when the TATA box of *UGT1A1* is *UGT1A1*28* [$(TA)_7$], whereas when it is *UGT1A1*1* [$(TA)_6$], the position –3279 in PBREM takes both T and G. These results show a clear contrast of ethnic differences compared to that reported among Caucasians and African-Americans [19].

### Irinotecan toxicity associated with T-3279G and *UGT1A1*28* genotype

We assessed toxicity profiles, including those for both *UGT1A1*28* and T-3279G in PBREM. Distributions of *UGT1A1*28* among the patients who experienced severe toxicity and those who did not were significantly different ($P < 0.001$) (Table 2), and univariate analysis showed a significant association between the variants for *UGT1A1*28* and the occurrence of severe toxicity (OR 5.33; 95% CI 2.02–14.1). Twenty-three of our 119 patients who had both T-3279G and *UGT1A1*28* variants (Table 3) were 6.2-fold more likely to encounter severe toxicity compared to homozygotes of the reference allele (OR 6.22; 95% CI 2.16–17.9). The patients with a single variant for T-3279G were 1.24-fold more likely to encounter severe toxicity compared to homozygotes of the reference allele (OR 1.24; 95% CI 0.38–4.02). However, multiple logistic regression analysis showed that the association between the T-3279G genotype and irinotecan toxicity did not persist after including *UGT1A1*28* (OR 0.95; 95% CI 0.18–5.05), although the association between *UGT1A1*28* and irinotecan toxicity did remain after including T-3279G variants (OR 6.84; 95% CI 1.86–25.2). In addition, the stepwise procedures identified female gender and irinotecan plus other drugs (apart from platinum) as important variables for the occurrence of severe toxicity besides the *UGT1A1*28* genotype. After adjusting for these two variables, the importance of the *UGT1A1*28* genotype was still verifiable. These findings suggest that *UGT1A1*28* might be a stronger predictive factor for severe irinotecan toxicity than T-3279G variants.

**Table 2 Associations of *UGT1A1* promoter genotypes and severe toxicity**

| | Leukopenia (grade 4) and/or diarrhoea (grade 3 or worse)[a] | | *P* |
|---|---|---|---|
| | Experienced ($n=27$) | Not experienced ($n=92$) | |
| T-3279G | | | 0.012 |
| T/T | 10 (37%) | 58 (63%) | |
| T/G | 10 (37%) | 27 (29%) | |
| G/G | 7 (26%) | 7 (8%) | |
| *UGT1A1*28* | | | <0.001 |
| $(TA)_6/(TA)_6$ | 15 (56%) | 80 (87%) | |
| $(TA)_6/(TA)_7$ | 8 (30%) | 9 (10%) | |
| $(TA)_7/(TA)_7$ | 4 (15%) | 3 (3%) | |

[a]Japan Society for Cancer Therapy criteria.

**Table 3 Associations of T-3279G and *UGT1A1*28* genotypes with severe toxicity**

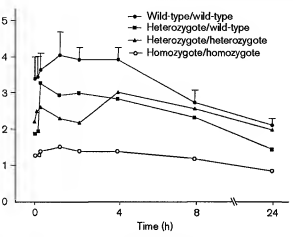
| | | Leukopenia (grade 4) and/or diarrhoea (grade 3 or worse)[a] | |
|---|---|---|---|
| | | Experienced ($n=27$) | Not experienced ($n=92$) |
| T-3279G | *GT1A1*28* | | |
| T/G or G/G | $(TA)_6/(TA)_7$ or $(TA)_7/(TA)_7$ | 12 | 11 |
| T/G or G/G | $(TA)_6/(TA)_6$ | 5 | 23 |
| T/T | $(TA)_6/(TA)_7$ | 0 | 1 |
| T/T | $(TA)_6/(TA)_6$ | 10 | 57 |

[a]Japan Society for Cancer Therapy criteria.

### Glucuronidation of irinotecan associated with T-3279G and *UGT1A1*28* genotype

The time-course of the metabolic ratio (SN-38G/SN-38 in plasma concentration) was measured in the patients

Fig. 1




Time-course of the metabolic ratios (SN-38G/SN-38) in patients with each genotype of the phenobarbital-responsive enhancer module gene and *UGT1A1*. There was one patient with double variants for T-3279G homozygote and *UGT1A1*28* homozygote (homozygote/homozygote, open circle), one patient with double variants for T-3279G heterozygote and *UGT1A1*28* heterozygote (heterozygote/heterozygote, closed triangle), one patient with single variant for T-3279G heterozygote and *UGT1A1* reference alleles (heterozygote/wild-type, closed square), and three patients with reference alleles (wild-type/wild-type, closed circle, mean ± SEM).

with T-3279G and *UGT1A1*28* genotype (Fig. 1). The metabolic ratios of SN-38G/SN-38 were lowest in the patient carrying double-variants for T-3279G homozygote and *UGT1A1*28* homozygote. Furthermore, those in the patient with single variant for T-3279G heterozygote (heterozygote/wild-type in Fig. 1) ranged between those with double-homozygote variants (homozygote/homozygote in Fig. 1) and those with reference alleles (wild-type/wild-type). The AUC ratios for SN-38 glucuronidation were measured. The $AUC_{SN-38G}/AUC_{SN-38}$ ratio for the patient carrying double-variants for T-3279G homozygote and *UGT1A1*28* homozygote (homozygote/homozygote in Fig. 1) was 1.058, that for the patient with double-variants for T-3279G heterozygote and *UGT1A1*28* heterozygote (heterozygote/heterozygote in Fig. 1) was 1.836, that for the patient with single-variant for T-3279G heterozygote (heterozygote/wild-type in Fig. 1) was 1.736, and that with reference alleles (wild-type/wild-type) was 2.353 (2.291–3.049) (median and interquartile range). These findings suggest that the polymorphism of T-3279G may affect the glucuronidation of SN-38, although it does not cause a marked alteration.

## Discussion

The interpatient variability of a given individual's drug response and resulting toxicity is a major problem in cancer chemotherapy. Such variability may be due to drug–drug interactions, the patient's age, renal and liver function, performance status, or concomitant illnesses. An even more important factor is the genetic variation in genes for drug-metabolizing enzymes. For example, severe and potentially fatal toxicity have been reported among patients with the genetic polymorphism of thiopurine *S*-methyltransferase (*TPMT*) or dihydropyrimidine dehydrogenase (*DPD*) [20–22]. Recently, we and another group suggested that the determination of *UGT1A1* genotypes, especially *UGT1A1*28*, might be clinically useful for predicting severe irinotecan toxicity in cancer patients [11–12]. Myelosuppression and diarrhoea occurred in patients treated with irinotecan who had an inherited deficiency in glucuronidation from the promoter polymorphism of *UGT1A1*, *UGT1A1*28*. In addition, we previously reported the relationship between the multiple variant genotypes (*UGT1A1*6*, *UGT1A1*27*, *UGT1A1*29* and *UGT1A1*7* in addition to *UGT1A1*28*) and the severe toxicity of irinotecan. *UGT1A1*6* has been reported to be the prevalent *UGT1A1* variant that contributes to nonphysiological hyperbilirubinemia in Asian populations. According to expression studies performed *in vitro*, *UGT1A1*6* in the homozygous and heterozygous genetic states decreases the enzyme activity to 32% and 60% of control, respectively. However, multivariate analysis suggested no statistical association of *UGT1A1*6* with the occurrence of severe irinotecan toxicity. All three patients heterozygous for *UGT1A1*27* encountered severe toxicity, although they coexist with a variant sequence in the promoter *UGT1A1*28*. None had *UGT1A1*29* or *UGT1A1*7*. Multivariate analysis suggested that only *UGT1A1*28* would be a significant risk factor for severe toxicity by irinotecan ($P < 0.001$; OR 7.23; 95% CI 2.52–22.3) [12]. Because 14 patients did not have the *UGT1A1*28* genotype among the 26 patients who had both grade 4 leukopenia and grade 3 or worse diarrhoea [12], it might be due to some as yet undiscovered variant UGT alleles or other genetic drug-metabolizing polymorphisms by reason of their severe toxicity, indicating the need for further investigations to discover other genetic variations associated with severe toxicity.

*UGT1A1*28* is also involved in the pathogenesis of Crigler–Najjar type II disease with hyperbilirubinemia in infants. Phenobarbital is known to induce a transcription of the *UGT1A1* gene in liver *in vivo*, and phenobarbital treatment has been shown to dramatically reduce the hyperbilirubinemia in the disease [14]. Sugatani *et al.* [13] recently identified PBREM in the *UGT1A1* promoter approximately 3 kb upstream of the TATA box. PBREM was activated by hCAR binding to gtNR1, one of the putative nuclear receptor motifs. A mutation of gtNR1 resulted in an 80% decrease in hCAR-dependent enhancer activity, whereas mutations of either NR3 or NR4 decreased it by approximately 50%. When all three motifs were simultaneously mutated, the 290-bp DNA

completely lost its enhancer activity. These results indicate that all three motifs might be required to confer full enhancer activity upon the *UGT1A1* promoter. Furthermore, Sugatani *et al.* [15] reported a T-3279G variant in NR3, and Innocenti *et al.* [19] showed that SN-38 glucuronidation was reduced in a T-3279G variant compared to homozygotes for the reference allele, although the reduction was without statistical significance. New polymorphisms, C-3440A and T-3401C, have been reported in the PBREM among Caucasian and African-American populations, although it is not yet clear whether these polymorphisms affect the enhancer activity of the *UGT1A1* promoter [19].

In the present study, the polymorphism of T-3279G was detected in the NR3 domain of PBREM, and the allele frequency of the variant T-3279G was 0.273 in Japanese cancer patients. The genotype frequencies of TT, TG, and GG in T-3279G were 0.53, 0.40 and 0.07, respectively, which is similar to the results obtained from healthy Japanese subjects, although the number of subjects was small ($n = 27$) [15]. As reported previously, the genotype frequencies of TT, TG and GG were 0.24, 0.58, 0.18 for Caucasians and 0.03, 0.24 and 0.73 for African-Americans, respectively [19]. These results show the clear contrast of ethnic differences in genetic variation: GG at position –3279 is the major genotype in African-Americans, whereas TT is the major one in Asians, and Caucasians are inbetween. We could not detect any polymorphisms in, or between, the NR4 or gtNR1 domains, including variants of C-3440A and T-3401C.

We assessed the relationship between T-3279G variants and irinotecan toxicity. Distributions of T-3279G were significantly different between the patients who experienced severe toxicity and those who did not. Although simple logistic regression analysis showed a significant association between the homozygotes for T-3279G and the occurrence of severe toxicity, multiple logistic regression analysis revealed that such an association did not persist after including *UGT1A1*28*. This might be due to the highly significant linkage disequilibrium between the T-3279G and *UGT1A1*28* polymorphisms. We showed that the position –3279 in PBREM preferentially takes G when the TATA box of *UGT1A1* is *UGT1A1*28* [$(TA)_7$], with the result that only one patient showed a single variant for *UGT1A1*28*. When the TATA box is *UGT1A1*1* [$(TA)_6$], the position –3279 in PBREM takes both T and G. These results also reveal ethnic differences (i.e. the linkage disequilibrium showed only low levels of significance between $(TA)n$ and the position –3279 in Caucasians and African-Americans [19]). Furthermore $(TA)_6$ is found with T at –3279 whereas $(TA)_7$ is found with the alternative alleles at –3279 [19].

We demonstrated that the patients who had double variants for T-3279G and *UGT1A1*28* were 6.2-fold more likely to encounter severe irinotecan toxicity compared to those with homozygotes for the reference allele. This finding was also supported by the results from the measurement of the metabolic ratios of SN-38 to SN-38G. We could not determine whether the existence of double variants for T-3279G and *UGT1A1*28* increased the occurrence of severe toxicity more than a single variant for *UGT1A1*28*. In our analysis, the only patient with a single variant for *UGT1A1*28* did not have severe toxicity, and we could not obtain the SN-38G/SN-38 metabolic ratios of the patient. We also showed that patients with a single variant for T-3279G were 1.24-fold more likely to encounter severe toxicity than homozygotes of the reference allele. The finding that the metabolic ratios of the patient with single variant for T-3279G heterozygote were lower than those with reference alleles might support the role of T-3279G polymorphism for the glucuronidation of SN-38.

In conclusion, we suggest that a highly significant linkage disequilibrium exists between T-3279G and *UGT1A1*28* polymorphisms, and that the variants of T-3279G and *UGT1A1*28* cooperatively decrease the transcriptional activity of the *UGT1A1* promoter. Therefore, the determination of both T-3279G and *UGT1A1*28* genotypes might be clinically useful in predicting severe irinotecan toxicity in cancer patients.

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# Combined Test for UGT1A1 −3279T→G and A(TA)$_n$TAA Polymorphisms Best Predicts Gilbert's Syndrome in Italian Pediatric Patients

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## ABSTRACT

**Gilbert's syndrome is a common hereditary chronic or recurrent, mild unconjugated hyperbilirubinemia. Polymorphisms in the bilirubin uridine diphosphate glucuronosyl transferase gene (*UGT1A1*) causing a decreased enzyme activity are associated with susceptibility to the syndrome. Homozygosity for TA$_7$ allele of the A(TA)$_n$TAA promoter polymorphism is found in the majority of Caucasian patients. We sought to investigate the role of three UGT1A1 polymorphisms (A[TA]$_n$TAA, −3279T→G, and G71R) in the susceptibility to Gilbert's syndrome in 53 Italian pediatric subjects compared to 83 unaffected controls. Carriage of two TA$_n$ risk alleles (TA$_7$ and TA$_8$) and −3279G homozygosity were similarly associated with hyperbilirubinemia (odds ratio [OR] = 11.59, 95% confidence interval [CI] = 4.80–27.98; $p < 0.001$, and OR = 11.51, 95% CI = 5.06–26.19; $p < 0.001$, respectively). Homozygosity for both TA$_7$ and −3279G was associated with the highest relative risk estimate (OR = 19.23, 95% CI = 7.34–50.4; $p < 0.001$), but a significant association was found also for TA$_7$ heterozygosity combined with −3279G/G genotype (OR = 7.98, 95% CI = 2.54–25.06; $p < 0.001$). The G71R variant was found only in two controls. Our results demonstrate that genotyping of both UGT1A1 A(TA)$_n$TAA and −3279T→G polymorphisms best defines genetic susceptibility to Gilbert's syndrome in Caucasian pediatric patients, and the TA$_7$ heterozygous genotype combined with homozygosity for the −3279G allele can also be associated with pediatric mild hyperbilirubinemia.**

## INTRODUCTION

GILBERT'S SYNDROME is characterized by a hereditary chronic or recurrent, mild unconjugated hyperbilirubinemia with otherwise normal liver function and hematological tests (Berk and Noyer 1994), with a prevalence reaching up to 10% in the general population (Bosma 2003). The increase of serum bilirubin is variable, ranging from normal to approximately 5 mg/dl (approximately 90 $\mu$M). Patients are usually identified by elevated serum unconjugated bilirubin levels on screening blood tests or after the occurrence of mild jaundice, especially on fasting, stress, or intercurrent infection. The syndrome is usually benign and no treatment is required; however, patients can complain of associated symptoms such as vertigo, headache, fatigue, abdominal pain, nausea, diarrhea, constipation, and loss of appetite (Cleary and White 1993). Moreover, an increased prevalence of idiopathic cholelithiasis has been recently reported in pediatric patients (Kitsiou-Tzeli et al. 2003).

Gilbert's syndrome is caused by polymorphisms in the *UGT1A1* gene causing a decreased enzyme activity and a reduced glucuronidation of bilirubin. In Caucasian patients, the most frequent genotype is the homozygous TA$_7$ allele at the A(TA)$_n$TAA repeat in the promoter TATA box of the gene (Bosma et al. 1995; Beutler et al. 1998). Another variant, TA$_8$, similar to TA$_7$ in reducing the transcriptional activity of UGT1A1, is common in people of African ancestry, but rare in Caucasians (Beutler et al. 1998; Iolascon et al. 1999; Tsezou et al. 2000; Coelho et al. 2004). A second polymorphism in UGT1A1 exon 1, G71R, has been detected with high frequency in East Asian hyperbilirubinemic patients (Akaba et al. 1998;

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Maruo et al. 1999), but only occasionally in Caucasians with hereditary hyperbilirubinemia (Kraemer and Klinker 2002; Sava and Kraemer 2005). Furthermore, in a small number of patients and controls from Japan and Argentina, the $TA_7$ allele has been found in linkage with −3279T→G, a third polymorphism frequent in the Japanese population mapping to the phenobarbital responsive enhancer module of UGT1A1 (Sugatani et al. 2002; Maruo et al. 2004). Because limited data are available on the −3279T→G and G71R variants in Caucasians, we sought to analyze all three polymorphisms in a group of Italian pediatric patients with unconjugated hyperbilirubinemia compatible with a diagnosis of Gilbert's syndrome.

## MATERIALS AND METHODS

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was performed according to the recommendations of the Ethics Committee of IRCCS "Bambino Gesù" Children's Hospital.

### *Subjects*

We included in the study 53 untreated consecutive patients (31 males and 22 females, M:F ratio = 1.41:1), with mean age of 10.9 (±5.3) years (range, 2–18). Patients were recruited at the hepatology unit of "Bambino Gesù" Children's Hospital from June 2004 to April 2005. Gilbert's syndrome was clinically diagnosed through the detection of either slightly elevated serum total bilirubin (>1 mg/dl or 17.1 $\mu M$) or elevated serum levels after fasting (1.4 to 6.1 mg/dl or 23.9 to 104.3 $\mu M$). Except for the unconjugated hyperbilirubinemia, blood values of all patients were normal.

As a control group, we examined 83 unaffected subjects (48 males and 33 females, M/F ratio = 1.45:1), with mean age of 11.1 (±8.0) years (range, 1–34) and no known history of jaundice. All subjects had serum bilirubin less than 1 mg/dl (17.1 $\mu M$), which represents the upper normal limit in our laboratory.

### *Genotyping*

After obtaining informed consent from the parents of each patient, genomic DNA was extracted from peripheral blood samples. The fragments encompassing the three polymorphisms, $A(TA)_nTAA$, −3279T→G, and 211G→A (G71R), were polymerase chain reaction (PCR)-amplified in a 25-µl reaction mixture containing 50 ng genomic DNA, 0.2 $mM$ of each primer (MWG-Biotech AG, Ebersberg, Germany), 3 $mM$ $MgCl_2$ (2.5 $mM$ for G71R), 0.2 $mM$ dNTPs, 0.75 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 1× GeneAmp Buffer II (Applied Biosystems). Conditions were 95°C for 10 min followed by 45 cycles (32 for $A[TA]_8TAA$) of 95°C for 30 sec, annealing temperature (Table 1) for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 7 min. For molecular analysis of $A(TA)_nTAA$, fluorescence-labeled PCR products were separated by automated capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and analyzed with GeneScan and Genotyper software (Applied Biosystems). For genotyping of −3279T→G and G71R variants, PCR-amplified fragments were analyzed by pyrosequencing on a PSQ96MA instrument (Biotage AB, Uppsala, Sweden) following the manufacturer's protocols. Nucleotide dispensation orders were CGTCGACAG for −3279T→G and GTCTCGTCT for G71R. Oligonucleotide primer sequences for PCR and pyrosequencing are detailed in Table 1.

### *Statistical analysis*

Genotype and allele frequencies in cases and controls were compared by $\chi^2$ test or Fisher's exact test. OR and 95% CI were calculated to estimate relative risks. A $t$ test was performed to compare mean bilirubin values. Construction of haplotypes was performed using the algorithm implemented in PHASE version 2 (Stephens et al. 2001; Stephens and Donnelly 2003). Haplotype frequencies and linkage disequilibrium ($D'$ and $r^2$) measures were calculated using HAPLOVIEW (Barrett et al. 2005).

## RESULTS

### *Single polymorphism analysis*

Comparison for genotype counts at $A(TA)_nTAA$ and −3279T→G variants are presented in Table 2.

Among the 53 hyperbilirubinemic subjects, 30 (56.6%) were homozygous for the $A(TA)_7TAA$ allele and 1 (1.9%) had a $TA_7/TA_8$ heterozygous genotype. A total of 31 patients carried

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDE PRIMERS FOR PCR AND PYROSEQUENCING REACTIONS

| *Polymorphism* | *Primer* | *Sequence (5′→3′)* | *Fragment size (nt)* | $T_A$ (°C) |
|---|---|---|---|---|
| $A(TA)_6TAA$ | PCR forward | TCTGAAAGTGAACTCCCTGCTACC | 154[a] | 60 |
| | PCR reverse | FAM-ATGGCGCCTTTGCTCCCTG | | |
| −3279T→G | PCR forward | ACGCAATGAACAGTCATAGTA | 168 | 59 |
| | PCR reverse pyrosequencing | Biotin-AAGCTCATTCCTCCTCTCTAG | | |
| | | CCAAGGGTAGAGTTCAGT | | |
| 211G→A (G71R) | PCR forward | AGAGGGGACATGAAATAGTT | 84 | 55 |
| | PCR reverse pyrosequencing | Biotin-GGTACGTCTTCAAGGTGTAA | | |
| | | CTTCAAGGTGTAAAATGC | | |

[a]Size for $A(TA)_6TAA$ allele.

nt, nucleotides; $T_A$, annealing temperature for PCR reactions; PCR, polymerase chain reaction.

TABLE 2. GENOTYPES AT UGT1A1 A(TA)$_n$ TAA AND −3279T→G POLYMORPHISMS IN 53 HYPERBILIRUBINEMIC PATIENTS AND 83 UNAFFECTED CONTROLS

| | | Genotype counts (%) | | | | |
|---|---|---|---|---|---|---|
| *Polymorphism* | *Group* | *−/−* | *−/+* | *+/+* | *OR (95% CI)*[a] | p[a] |
| A(TA)$_n$TAA[b] | Controls | 37 (44.6) | 37 (44.6) | 9 (10.8) | 1 | |
| | Patients | 0 | 22 (41.5) | 31 (58.5) | 11.59 (4.80–27.98) | <0.001 |
| −3279T→G | Controls | 23 (27.7) | 41 (49.4) | 19 (22.9) | 1 | |
| | Patients | 0 | 12 (22.6) | 41 (77.4) | 11.51 (5.06–26.19) | <0.001 |

[a]OR and *p* values for +/+ genotypes compared to −/− and −/+ combined genotypes.
[b]− indicates $TA_6$ allele; + indicates $TA_7$ or $TA_8$ alleles.
OR, odds ratio; CI, confidence interval.

two copies of Gilbert-associated alleles ($TA_7$ or $TA_8$) compared to 9 of 83 controls (OR = 11.59, 95% CI = 4.80–27.98; $p < 0.001$). No hyperbilirubinemic subject was homozygous for the $TA_6$ allele compared to 37 controls. The combined frequency of risk alleles ($TA_7$ or $TA_8$) was 79.2% in patients compared to 33.1% in controls ($p < 0.001$).

Similarly, the frequency of −3279T→G homozygous mutant genotypes was higher in patients than in controls, with an OR of 11.51 (95% CI = 5.06–26.19; $p < 0.001$). Homozygosity for the −3279T allele was detected in 23 controls and in no patients. The allele frequency of −3279G was 88.7% in patients compared to 47.6% in controls ($p < 0.001$).

Only two control subjects (2.4%) and no patients were heterozygous for G71R.

### *Combined −3279T→G and A(TA)$_n$TAA analysis*

A(TA)$_n$TAA and −3279T→G polymorphisms were in linkage disequilibrium (D′ = 0.98, 95% CI = 0.89–1.0; $r^2 = 0.57$) in the whole population, as well as in each group of patients and controls (data not shown). Haplotype construction at the two polymorphic loci revealed three haplotypes with frequency greater than 1%, namely T6 (36%), G7 (50%), and G6 (12.9%), representing 98.9% of chromosomes.

The analysis of haplotype distribution revealed that two genotypes, G7 homozygote and G6/G7 compound heterozygote, were more frequent in hyperbilirubinemic cases than in controls (Table 3). Homozygosity for the G7 haplotype was associated with hyperbilirubinemia with an OR = 19.23 (95% CI = 7.34–50.4; $p < 0.001$), but interestingly also the G6/G7 genotype showed a significant association (OR = 7.98, 95% CI = 2.54–25.06; $p < 0.001$). In total, 41 of 53 (77.4%) patients carried one of the two associated genotypes compared to 16 of 83 (19.3%) controls (OR = 14.31, 95% CI = 6.16–33.25; $p < 0.001$).

In line with these findings, among those heterozygotes for the $TA_7$ allele the frequency of −3279G homozygous genotypes was significantly higher in patients (10/22; 45.5%) than in controls (7/37; 18.9%), with an OR of 3.57 (95% CI = 1.10–11.57; $p = 0.04$ at Fisher's exact test).

### *Genotype–phenotype correlation*

Among hyperbilirubinemic subjects, the mean value of bilirubinemia was higher in G7 homozygotes than in other haplotypes (1.98 ± 0.51 mg/dl versus 1.43 ± 0.31 mg/dl, $p < 0.001$). The difference between the two mean values was 0.55 mg/dL (95% CI: 0.32–0.77). The other risk haplotype (G6/G7) was not associated with significantly higher levels of bilirubin.

## DISCUSSION

This is the first study to analyze the frequency of the UGT1A1 A(TA)$_n$TAA and −3279T→G variants in a large group of hyperbilirubinemic Caucasian pediatric patients. Although the two polymorphisms were in linkage disequilibrium, this was not sufficient to allow for genotypes at one locus to be fully representative of genotypes at the other. All $TA_7$ homozygous subjects were also homozygous for the −3279G al-

TABLE 3. DISTRIBUTION OF $TA_7$ GENOTYPES COMBINED WITH −3279G HOMOZYGOSITY IN 53 HYPERBILIRUBINEMIC PATIENTS AND IN 83 UNAFFECTED CONTROLS

| *Genotypes* | *Patients (%)* | *Controls (%)* | *OR (95% CI)* | p |
|---|---|---|---|---|
| G7/G7 | 31 (58.5)[a] | 9 (10.8) | 19.23 (7.34–50.4) | <0.001 |
| G6/G7 | 10 (18.9) | 7 (8.4) | 7.98 (2.54–25.06) | <0.001 |
| Other genotypes | 12 (22.6)[b] | 67 (80.7)[c] | 1 | |

[a]The single patient with G7/G8 genotype was included in G7/G7 subjects.
[b]Twelve patients were T6/G7 compound heterozygous.
[c]Among unaffected controls the T6/G7 genotype was found in 29 (34.9%) individuals, T6/T6 in 22 (26.5%), T6/G6 in 12 (14.5%), G6/G6 in 3 (3.6%), and T6/T7 in 1 (1.2%) subjects.
OR, odds ratio; CI, confidence interval.

lele, in agreement with previously reported results (Maruo et al. 2004), and homozygosity for the G7 haplotype was associated with the highest relative risk estimate. Interestingly, homozygosity for the −3279G allele was significantly associated with hyperbilirubinemia also when combined with the $TA_7$ heterozygous genotype (G6/G7 haplotype). These results demonstrate that the combination of −3279G homozygosity with a single $TA_7$ allele can be considered as an additional Gilbert-associated genotype in Caucasian pediatric patients, as recently suggested in a study on Portuguese adolescent and adult Gilbert patients (age range, 14–45 years) (Costa et al. 2005).

Both $A(TA)_nTAA$ and −3279T→G polymorphisms localize in regulatory sequences 5′ of the UGT1A1 gene and cause decreased transcription (Bosma et al. 1995; Sugatani et al. 2002). It is still controversial whether $TA_7$ homozygosity is sufficient to explain the decrease of bilirubin UDP-glucuronosyltransferase activity to approximately 30% of normal value, as observed in Gilbert patients (Arias and London 1957; Black and Billing 1969). Although *in vitro* expression studies indicated that the transcriptional activity of the $TA_7$ promoter might be even lower than 30% (Bosma et al. 1995; Ciotti et al. 1998), other studies reported a less significant reduction to approximately 60%–80% of normal values (Ueyama et al. 1997; Beutler et al. 1998). In addition, a correlation between the $A(TA)_nTAA$ polymorphism and human liver UGT1A1 enzyme activity showed the lowest activity in subjects with a $TA_7$ homozygous genotype (48% compared to $TA_6$ homozygotes) and intermediate values (63%) for heterozygous $TA_6/TA_7$ subjects (Raijmakers et al. 2000). Similarly, the −3279T→G polymorphism is known to decrease the transcriptional activity to only 62% of normal (Sugatani et al. 2002). Thus, although each polymorphism would only induce a minor reduction in glucuronidation, it can be speculated that their associated genotypes generate a combined effect lowering the enzymatic activity to the Gilbert characteristic level. Further biochemical studies are needed to demonstrate whether the $TA_7$ heterozygous genotype combined with −3279G homozygosity may also decrease the UGT1A1 transcriptional activity over a threshold of disease.

UGT1A1 is involved also in the metabolism of the antitumor drug irinotecan, which is widely used in the treatment of patients with colorectal or lung cancer. The reduced UGT1A1 activity due to the $A(TA)_7TAA$ and −3279G polymorphisms decreases the biotransformation of the active metabolite SN-38 into the inactive SN-38 glucuronide (SN-38G), leading to increased susceptibility to severe irinotecan toxicity. In fact, cancer patients with both −3279G and $TA_7$ alleles showed lower plasma SN-38G/SN-38 ratios and the greatest relative risk to encounter severe adverse effects, such as leukopenia and diarrhea (Ando et al. 1998; Iyer et al. 1998; Kitagawa et al. 2005).

Other UGT1A1 genotypes have been rarely reported in Europeans. $TA_7/TA_8$ compound heterozygosity has been previously described in two Italian relatives (an 8-year-old girl and her mother) and in a 5-year-old Portuguese girl (Iolascon et al. 1999; Coelho et al. 2004), while a 3-year-old Greek boy with Gilbert's syndrome was found to be $TA_6/TA_8$ heterozygous (Tsezou et al. 2000). Among our patients, only one carried the $TA_7/TA_8$ genotype, confirming the rarity of $TA_8$ allele in Caucasians. In fact, this allele is more common in African populations, reaching a frequency of 6.9% (Beutler et al. 1998). The UGT1A1 G71R polymorphism is another noticeable example of allele frequency variability in different populations. This variant is common in East Asians, with frequencies of 13%–16% in Japanese and 23% in Chinese and Korean populations (Akaba et al. 1998; Maruo et al. 1999), but very rare in Europe, where one G71R heterozygous patient with Gilbert's syndrome and a single G71R/Y486D compound heterozygous patient with Crigler-Najjar syndrome type II have been described in Germany (Kraemer and Klinker 2002; Sava and Kraemer 2005). Homozygosity for G71R decreases enzyme activity to approximately 30% of normal, a level compatible with development of Gilbert's syndrome, while heterozygosity is associated with about 60% enzymatic activity, which is usually adequate to maintain the bilirubin metabolism within normal limits (Yamamoto et al. 1998). In line with these findings, we found two healthy controls and no patients who were heterozygous for the G71R variant.

In conclusion our results demonstrate that in addition to $A(TA)_7TAA$ homozygosity, the $TA_7$ heterozygous genotype combined with homozygosity for the −3279G allele also associates with genetic susceptibility to Gilbert's syndrome in Caucasians. We propose that genotyping of −3279T→G should be included in routine molecular testing for this condition.

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# Study of the genetic determinants of *UGT1A1* inducibility by phenobarbital in cultured human hepatocytes

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**UGT1A1 is induced by phenobarbital. We investigated whether three common *UGT1A1* variants are associated with the variability in *UGT1A1* inducibility. Human hepatocytes were incubated with 2 mM phenobarbital for 2 and 6 days followed by 5 μM SN-38 (1 h), a UGT1A1 probe. SN-38 glucuronidation in the cell media was measured by high-performance liquid chromatography. Three *UGT1A1* promoter variants [−53(TA)$_{6>7}$, −3156G>A and −3279T>G] were genotyped. Significant induction of UGT1A1 catalytic activity was observed in 82% and 100% of the cultures treated with phenobarbital for 2 days (median fold-induction = 1.6, range 1.3–2.8; $n$ = 28) and 6 days (median fold-induction = 2.8, range 1.6–6.4; $n$ = 16), respectively. After 2 days of treatment, a negative correlation was observed between the UGT1A1 basal activities and the fold-induction (Spearman $r$ = −0.52, $P$ < 0.005). By contrast, the UGT1A1 activities in the basal and induced states were highly correlated (Spearman $r$ = 0.95, $P$ < 0.0001). Similar results were observed after 6 days of treatment. The allele frequencies were not significantly different between induced ($n$ = 22) and non-induced preparations ($n$ = 6) ($P$ > 0.05). The fold-induction was not associated with any variants ($P$ > 0.05). The basal and induced activities were correlated with −53(TA)$_{6>7}$ (and with −3156G>A due to almost complete linkage with the −53 indel) ($P$ = 0.001). No association was found with the −3279T>G single nucleotide polymorphism ($P$ > 0.05). The indel at −53 affects the basal phenotype and appears to limit the hepatocyte capability of maximal induction after phenobarbital. However, variants at −53, −3156 and −3279 are not associated with variability in *UGT1A1* inducibility. *Pharmacogenetics and Genomics* 16:79–86 © 2006 Lippincott Williams & Wilkins.**

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## Introduction

Biotransformation by glucuronidation is a common metabolic reaction that improves the hydrophilicity of endogenous and exogenous compounds, facilitating their biliary and urinary excretion. Glucuronidation is catalysed by the microsomal UDP-glucuronosyltransferase (UGT) enzymes and involves conjugation of uridine diphosphate glucuronic acid with acceptor molecules containing hydroxyl, carboxyl, sulfuryl, carbonyl or amino groups [1]. The human UGT enzymes are classified into UGT1 and UGT2 families based on similarities between their primary amino acid sequences [2]. The UGT1 family contains nine functional members (UGT1A1 and UGT1A3–10) derived from a single gene. Individual UGT1A isoforms have unique exon 1 sequences at the 5′ end locus which are independently transcribed and spliced to a shared set of exons 2–5 located at the 3′ end. Each *UGT1A* has a unique promoter with specific substrate preferences and tissue-specific expression [1,2].

*UGT1A1* is expressed in the human liver, bile ducts, stomach, colon and intestine [1]. This isoform is critical for the biliary excretion of bilirubin, a toxic breakdown product of heme metabolism [3]. UGT1A1 is also involved in the metabolism of steroid and thyroid hormones [4–7], anthraquinones and flavones [4], the topoisomerase I inhibitor SN-38 (the active metabolite of the anticancer agent irinotecan) [8], the topoisomerase II inhibitor etoposide [9] and the oral contraceptive steroid 17α-ethinylestradiol [10]. *UGT1A1* is induced by a wide variety of xenobiotics, including the anticonvulsants

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phenobarbital and phenytoin [11–13], the chemopreventive agent oltipraz [12–13], the carcinogen 3-methylcholantrene [11–13], the corticosteroid dexamethasone [14] and the dietary flavonoids chrysin and quercetin [15–16]. Exposure to *UGT1A1* inducers may lead to significant alteration in the metabolism of UGT1A1 substrates and to drug–drug interactions.

A large number of genetic variants contribute to high inter-individual variability in the hepatic expression of *UGT1A1*. The *UGT1A1* promoter contains a polymorphic $(TA)_6TAA$ region that regulates basal enzyme expression. Homozygosity for the variant allele $(TA)_7TAA$ (*UGT1A1*28*) reduces UGT1A1 activity by 80% and is associated with Gilbert's syndrome (a mild form of unconjugated hyperbilirubinemia) in Caucasians [17,18]. Other genetic polymorphisms associated with reduced UGT1A1 activity include −3156G > A [located 117 bp upstream of the phenobarbital-responsive enhancer module (PBREM)] and −3279T > G (*UGT1A1*60*) (in the PBREM), both of which are found at high frequency in Caucasian, African-American and Asian populations [19–25]. In addition, *UGT1A1* has binding sites for nuclear receptors [the constitutive androstane receptor (CAR), the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) and the aryl hydrocarbon receptor (AhR)] that regulate the inducible expression of this gene by xenobiotics and flavones [26–31].

Induction of UGT1A1 glucuronidation has potential therapeutic implications, such as prevention of neonatal jaundice [32], treatment of unconjugated hyperbilirubinemia [18] and improvement of the therapeutic index of drugs that are detoxified by this isoform. It is not known whether *UGT1A1* polymorphisms affect inducibility. In the present study, we investigated whether the inter-individual variability in *UGT1A1* inducibility in response to phenobarbital is associated with the −53TA indel, and the −3156G > A and the −3279T > G polymorphisms, using primary human hepatocytes, the closest *in vitro* model to human liver [33].

## Materials and methods

### Chemicals

SN-38 and SN-38 glucuronide (SN-38G) were kindly provided by Pfizer, Inc. (Kalamazoo, Michigan, USA). Phenobarbital, camptothecin, sodium 1-heptanesulfonate and potassium phosphate monobasic were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). Hepatocyte maintenance media (HMM), insulin, dexamethasone, penicillin and amphotericin B were obtained from Cambrex Bioscience Walkersville Inc. (Walkersville, Maryland, USA). Streptomycin and bovine calf serum were purchased from Invitrogen Corporation (Carlsbad, California, USA). Acetonitrile, tetrahydrofuran, dimethyl sulfoxide (DMSO) and hydrochloric acid were obtained from VWR International, Inc. (Batavia, Illinois, USA). Thermosequenase DNA polymerase, thermosequenase reaction buffer, thermosequenase enzyme dilution buffer and the dideoxy nucleotide diphosphates were purchased from Amersham Biosciences (Bucks, UK).

### Hepatocyte isolation and culturing

Human liver tissue was obtained with the approval of Institutional Review Boards of the institutions involved in the Liver Tissue Procurement and Distribution System (Pittsburgh, Pennsylvania, USA), funded by NIH contract #N01-DK-9-2310. The demographics and medical history of the donors are shown in Table 1.

Hepatocytes were isolated from human donor livers by a three-step collagenase perfusion technique, as described previously [34]. Hepatocytes with a viability greater than 80% (assessed by a trypan blue exclusion method) ($n = 36$) were plated at 1.5 million cells per well on six-well culture plates previously coated with rat-tail collagen in HMM supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine calf serum ($HMM^+$). After cells attached for 4 h, the medium was replaced with serum-free $HMM^+$. After 24 h in culture, unattached cells were removed by gentle agitation. $HMM^+$ was replaced every 24 h. The cells were maintained in culture at 37°C in a humid atmosphere containing 5% $CO_2$ and 95% air.

### Treatment of hepatocytes

Preliminary experiments were performed to determine the optimum concentration of phenobarbital (range, 0.2–2 mM). At 48 h after plating, the media was aspirated and replaced with $HMM^+$ containing 2 mM phenobarbital (in 0.1% DMSO) for either 2 days ($n = 34$) or 6 days ($n = 16$). The control treatment was DMSO (0.1% final concentration). At the end of the exposure period, the cells were washed for 1 h with HMM and incubated for 1 h with the UGT1A1 substrate SN-38 (5 μM in 0.1% DMSO) [8]. Media (1 ml) was then removed and stored immediately at −80°C until analysis. Each treatment consisted of triplicate wells.

### SN-38 glucuronidation assay

UGT1A1 catalytic activity was assessed by measurement of SN-38 glucuronidation in hepatocyte media. Media aliquots (100 μl) were combined with 100 μl of internal standard (3 μM camptothecin in 0.01 N hydrochloric acid) and 70 μl of 0.3 N hydrochloric acid. After vortexing for 10 s, 100 μl aliquots were analysed by high-performance liquid chromatography (HPLC), as previously described [19]. Production of SN-38G was quantified using a standard curve containing known amounts of SN-38G (range 20–1200 ng/ml) and internal standard. The quantification limit of SN-38G was 20 ng/ml. The

Table 1 **Donor information**

| Donor HH # | Race[a] | Age[b] | Sex[c] | Smoking | Drug history |
|---|---|---|---|---|---|
| HH887 | NR | 19 | M | No | NR |
| HH888 | H | 5 | F | NR | Desmopressin acetate, dopamine, epinephrine |
| HH889 | C | 28 | M | Yes | Cefazolin, dexamethasone/hydrocortisone, dopamine, lorazepam, chronic marijuana, methylprednisolone |
| HH894 | C | 40 | M | No | Cimetidine/$H_2$ blocker, dopamine |
| HH899 | C | 23 | F | No | Barbiturates/phenytoin, cimetidine/$H_2$ blocker, desmopressin acetate, dopamine |
| HH965 | C | 17 | M | No | Dopamine |
| HH966 | C | 22 | M | No | Barbiturates/phenytoin, desmopressin acetate, fluoroquinolone |
| HH998 | C | 81 | M | Yes | Desmopressin acetate, dopamine, statin |
| HH1000 | C | 6 | M | No | Thyroid |
| HH1002 | C | 5 | M | No | Barbiturates/phenytoin, desmopressin acetate, dopamine, epinephrine, famotidine, furosemide, gentamicin, nafcillin, phenylephrine |
| HH1003 | C | 60 | F | No | NR |
| HH1012 | C | 74 | F | Yes | Desmopressin acetate, dopamine |
| HH1017 | AA | 1 | M | No | Cefazolin sodium, desmopressin acetate, dopamine, epinephrine |
| HH1018 | C | 12 | M | No | Desmopressin acetate, dopamine, epinephrine, mannitol |
| HH1032 | C | 15 | M | No | Barbiturates/phenytoin, desmopressin acetate, dopamine |
| HH1033 | C | 60 | M | Yes | Dexamethasone/hydrocortisone |
| HH1034 | AA | 41 | F | No | NR |
| HH1038 | NR | NR | NR | NR | NR |
| HH1038 | C | 47 | M | No | Desmopressin acetate, dexamethasone/hydrocortisone, erythromycin |
| HH1040 | C | 57 | M | No | Desmopressin acetate, dexamethasone/hydrocortisone, dopamine, erythromycin |
| HH1044 | C | 62 | M | No | Dopamine |
| HH1046 | C | 66 | M | NR | Dopamine, metoprolol tartrate, norepinephrine |
| HH1047 | C | 17 | M | No | NR |
| HH1051 | C | 74 | F | No | Desmopressin acetate, dopamine, flurazepam, levothyroxine sodium |
| HH1052 | C | 52 | F | Yes | Dopamine |
| HH1065 | H | 18 | M | No | Desmopressin acetate, dexamethasone/hydrocortisone, dopamine, erythromycin, ethanol |
| HH1069 | H | 41 | F | No | Desmopressin acetate, dexamethasone/hydrocortisone, dopamine |
| HH1073 | C | 52 | M | No | Dexamethasone/hydrocortisone, dopamine, thyroid |
| HH1078 | C | 61 | M | Yes | Cimetidine/$H_2$ blocker, dexamethasone/hydrocortisone, dopamine, fluoroquinolone |
| HH1092 | C | 41 | M | Yes | Dopamine, methylprednisolone sodium succinate, norepinephrine |
| HH1095 | C | 61 | M | No | Desmopressin acetate, dopamine, epinephrine |
| HH1100 | C | 69 | F | No | NR |
| HH1112 | C | 69 | F | NR | NR |
| HH1114 | C | 3 | F | NR | NR |
| HH1117 | C | 68 | F | NR | Clonidine hydrochloride, clopidogrel bisulfate, labetelol, simvastatin, verepamil |
| HH1118 | C | 73 | F | NR | Atenolol, dopamine, famotidine, furosemide, hydrochlorithiezide, peroxetine hydrochloride |

[a]C, Caucasian; H, Hispanic; AA, African-American; NR, no record given.
[b]NR, no record given.
[c]M, male; F, female.

intra-assay coefficients of variation (CVs) (range 0.9–3.5) and accuracy (range 97.7–104.3%) were determined by performing three measurements of seven SN-38G standards (range 20–1200 ng/ml) on the same day. Inter-assay precision (range 1.5–4.1) and accuracy (range 98.9–102.4%) were determined by performing assays of the standards in duplicate on three consecutive days.

### *UGT1A1* genotyping assays

The $-53(TA)_n$, $-3156G>A$ and $-3279T>G$ polymorphisms were genotyped in 28 samples (22 induced and six non-induced preparations) treated for 2 days with phenobarbital. The remaining samples ($n=6$, all induced) were not genotyped due to unavailability of DNA. Genotyping of $-53(TA)_n$ was performed by the polymerase chain reaction sizing, as previously described [19]. The $-3156G>A$ and $-3279T>G$ variants were genotyped by single base extension followed by denaturing HPLC [22]. The $-53(TA)_n$ polymorphism was assigned according to the number of $(TA)_n$ repeats in each allele (i.e. 6/6, homozygous for the $(TA)_6TAA$ allele, etc.).

### Statistical analysis

UGT1A1 catalytic activities were expressed as the mean concentration of SN-38G (ng/ml) from a single experiment performed in triplicate. Results were considered acceptable if the CVs of the triplicates were within 20%. We considered samples to be induced if the following two conditions were met: (i) the SN-38G levels in cells treated with phenobarbital increased by at least 25% compared to untreated cells and (ii) $P<0.05$ (unpaired Student's *t*-test). We expressed inducibility using the fold-induction, which was defined as the ratio of the concentration of SN-38G formed in the presence of phenobarbital to the control activity. The Spearman correlation coefficient was used to test the relationship between the basal SN-38G media concentrations and both the fold-induction and induced activities. The allele frequencies in the induced and non-induced preparations were compared using the chi-square test. Mann–Whitney and Kruskal–Wallis tests were used for two- and three-group comparisons, respectively. $P<0.05$ was considered statistically significant. Data and statistical analyses were performed using GraphPad Prism 4.00 for Windows (GraphPad Software Inc., San Diego, California, USA).

## Results

### Induction of UGT1A1 catalytic activity

In preliminary experiments to determine the optimal concentration of phenobarbital to use for induction, the median fold-increases in SN-38 glucuronidation in hepatocytes exposed to 0.2, 1 and 2 mM phenobarbital (for 2 days) were 1.1 (range 1.0–1.3; $n = 3$), 1.3 (range 1.1–1.4; $n = 3$) and 1.7 (range 1.2–2.4; $n = 5$), respectively. A concentration of 2 mM phenobarbital was chosen for subsequent experiments.

Induction of UGT1A1 activity following 2 days of phenobarbital treatment was observed in 82% (28 out of 34) of the hepatocyte preparations (median fold-induction = 1.6, range 1.3–2.8; $n = 28$) (Fig. 1). All preparations treated with phenobarbital for 6 days showed a significant increase in UGT1A1 activity (median fold-induction = 2.8, range 1.6–6.4; $n = 16$) compared to controls.

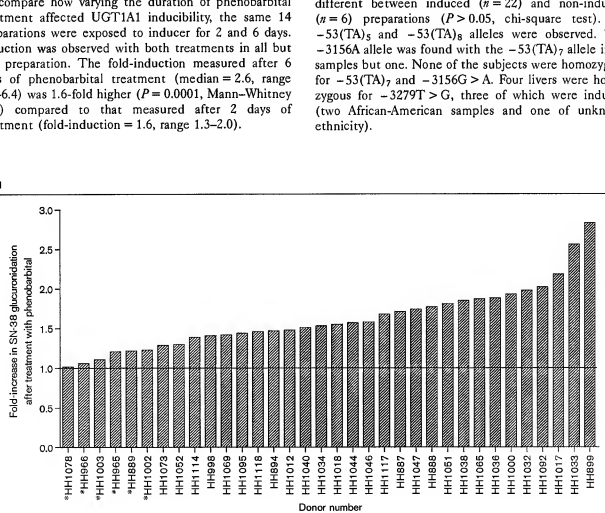
To compare how varying the duration of phenobarbital treatment affected UGT1A1 inducibility, the same 14 preparations were exposed to inducer for 2 and 6 days. Induction was observed with both treatments in all but one preparation. The fold-induction measured after 6 days of phenobarbital treatment (median = 2.6, range 1.6–6.4) was 1.6-fold higher ($P = 0.0001$, Mann–Whitney test) compared to that measured after 2 days of treatment (fold-induction = 1.6, range 1.3–2.0).

A wide range of UGT1A1 basal and induced activities was measured after 2 and 6 days of treatment (Table 2). After 2 days of treatment, the UGT1A1 activities in the basal and induced states were correlated (Spearman $r = 0.95$, $P < 0.0001$) (Fig. 2a). By contrast, a negative correlation was observed between the UGT1A1 basal activities and the fold-induction (Spearman $r = -0.52$, $P < 0.005$) (Fig. 2b). Similar correlations were observed after 6 days of treatment (data not shown). Ethnicity, gender, smoking status and drug history did not have an effect on the UGT1A1 basal activities (data not shown).

### Genotyping of *UGT1A1* variants and correlation with catalytic activities

The allele frequencies of the $-53(TA)_{6>7}$, $-3156G > A$ and $-3279T > G$ variants in the 28 genotyped samples from the day 2 treatments were 0.25, 0.23 and 0.43, respectively. Allele frequencies were not significantly different between induced ($n = 22$) and non-induced ($n = 6$) preparations ($P > 0.05$, chi-square test). No $-53(TA)_5$ and $-53(TA)_8$ alleles were observed. The $-3156A$ allele was found with the $-53(TA)_7$ allele in all samples but one. None of the subjects were homozygous for $-53(TA)_7$ and $-3156G > A$. Four livers were homozygous for $-3279T > G$, three of which were induced (two African-American samples and one of unknown ethnicity).

Fig. 1

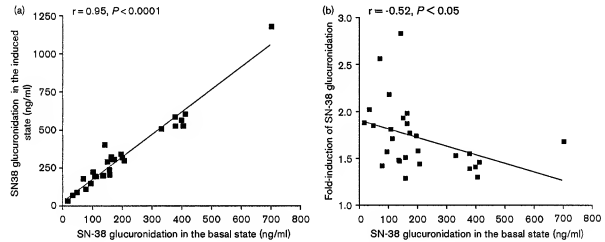



Effect of treating cultured primary human hepatocytes ($n = 34$) with 2 mM phenobarbital for 2 days on UGT1A1 activity. The horizontal line represents no change in UGT1A1 activity in phenobarbital-treated cells compared to untreated cells. *Samples that were not induced.

Table 2 **UGT1A1 glucuronidation activities measured in cultured primary human hepatocytes in the presence and absence of 2 mM phenobarbital**

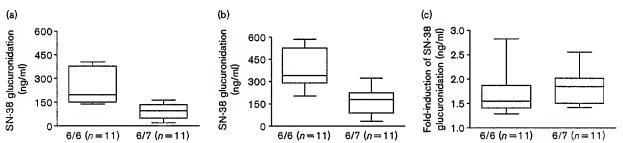
| | 2 days of treatment ($n=28$) | | | 6 days of treatment ($n=16$) | | |
|---|---|---|---|---|---|---|
| | Median SN-38 glucuronidation (ng/ml) | Range of SN-38 glucuronidation (ng/ml) | Fold-range of SN-38 glucuronidation | Median SN-38 glucuronidation (ng/ml) | Range of SN-38 glucuronidation (ng/ml) | Fold-range of SN-38 glucuronidation |
| Basal activity | 158 | 18–702 | 39 | 106 | 32–533 | 17 |
| Induced activity | 294 | 33–1178 | 36 | 376 | 97–916 | 9 |

**Fig. 2**




Correlation between the basal and induced SN-38 glucuronidation (a) and the basal SN-38 glucuronidation and the fold-induction (b) following 2 days of hepatocyte treatment using 2 mM phenobarbital. Each point represents the mean SN-38 glucuronide concentration (ng/ml) in the media of triplicate wells. Spearman correlation was performed.

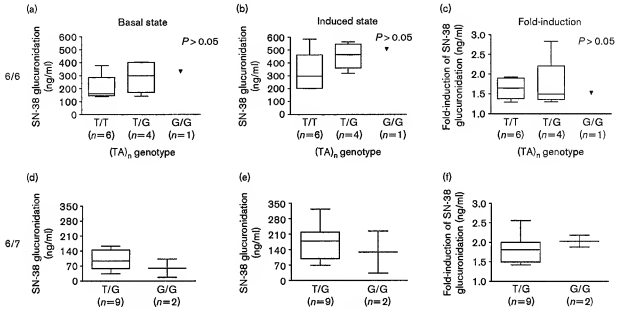
**Fig. 3**




Correlation between the $-53(TA)_{6>7}$ variant and SN-38 glucuronidation in (a) the basal state, (b) the induced state and (c) the fold-induction. Statistical significance was tested by the Mann–Whitney test.

The $-53$ 6/7 variant was not associated with the fold-induction ($P > 0.05$, Mann–Whitney test) but was correlated with a significant reduction in the basal and induced UGT1A1 activities ($P = 0.001$, Mann–Whitney test) (Fig. 3). Similar correlations were observed with the $-3156G > A$ variant (data not shown). No association was found between $-3279T > G$ and the fold-induction, the basal activities and the induced activities ($P > 0.05$, Kruskal–Wallis test). To investigate the possible effect of the $-3279T > G$ variant on the phenotypes without the potential confounding effect of the $-53$ variant, the correlation between $-3279T > G$ genotypes and

Fig. 4




Correlation between the −3279T>G variant and SN-38 glucuronidation in the basal state, the induced state and the fold-induction within the (a–c) 6/6 and (d–f) 6/7 genotypes. The Kruskal–Wallis test was used to test the association in the 6/6 samples. No statistical test was performed in the 6/7 samples due to the small number of samples ($n=2$) homozygous for −3279T>G.

phenotypes was then performed separately in the 6/6 ($n = 11$) and 6/7 ($n = 11$) samples. No effect on any phenotype was observed in the 6/6 ($P > 0.05$, Kruskal–Wallis test) (Fig. 4a–c) and 6/7 groups (Fig. 4d–f).

Similar correlations were observed when analysing data from Caucasians only ($n = 16$). The fold-induction was not significantly associated with any variant ($P > 0.05$, Mann–Whitney and Kruskal–Wallis tests). The $-53(TA)_{6>7}$ and −3156G > A variants were correlated with both basal and induced activities ($P < 0.05$, Mann–Whitney test) whereas no association was found with the −3279T > G polymorphism ($P > 0.05$, Kruskal–Wallis test).

## Discussion

This is the first study to investigate whether the inter-individual variation in UGT1A1 inducibility is associated with three common *UGT1A1* polymorphisms. Our experiments showed that variants at −53, −3156 and −3279 are not associated with inducibility of UGT1A1 activity in response to phenobarbital. The association of the $-53(TA)_{6>7}$ variant with a significant reduction in UGT1A1 activity is in agreement with previous reports [12,35]. The similar correlation observed between activity and the −3156G > A variant is probably due to the high extent of linkage disequilibrium between the −3156 and −53 sites in the present study (linkage was observed in all samples but one), as previously reported [19,22,24]. We were particularly interested in studying the effect of −3279T > G on inducibility because the variant resides in the DR3 element in the *UGT1A1* PBREM [19,26,31]. DR3 appears to be required for full enhancement activity although its function is still unknown [31]. We found no evidence of −3279T > G having a functional role in either the inducible or basal activity of UGT1A1. This polymorphism is associated with reduced transcriptional UGT1A1 activity and a significant increase in biliribin levels in Japanese and Koreans [20–21,23]. A functional effect in the Caucasian population was not found in this study, in agreement with a previous report [19]. Investigation of the functionality of −3279T > G (if any) in Caucasians requires further study utilizing a larger sample size.

The UGT1A1 catalytic activities observed in response to phenobarbital and the fold-induction were strongly pre-determined by the basal activities of each individual donor in such a way that donors with the highest basal activities had the highest induced activities but the lowest fold-induction. The variability in the activities decreased as the exposure to phenobarbital increased from 2 to 6 days, with the variation in the induced state

being more drastically reduced than the variability in the basal state. Although, under our experimental conditions, we did not observe a maximum level of UGT1A1 activity above which samples could not be induced, our observations suggest the existence of a threshold level above which the UGT1A1 enzyme cannot be induced. A similar relationship between the extent of induction and basal activities has been found in studies investigating the effect of rifampicin, paclitaxel and dexamethasone on CYP3A4, with the difference being that, in those studies, a maximum value of induced CYP3A4 catalytic activity was reached [36–38]. This is probably because CYP3A4 is induced to a higher extent than UGT1A1 [39]. On the other hand, it is possible that, if a threshold of inducibility exists for UGT1A1, it might become apparent in studies utilizing a more potent UGT1A1 inducer such as 3-methylcholantrene [12–13].

Previous studies using primary human hepatocytes to study the inducibility of UGT1A1 were performed using 2- and 3-day exposures to inducers [12–13,40]. Our research demonstrated that UGT1A1 activity is well maintained during culture of hepatocytes for 6 days, and that the magnitude of induction is significantly larger after 6 days of phenobarbital treatment compared to 2 days (1.63-fold). This is in agreement with enzyme induction being a slow regulatory process compared to inhibition [41].

The UGT enzymes in general lack substrate specificity, making it difficult to find specific probes. Hepatic enzymes catalysing the glucuronidation of SN-38 include UGT1A1, UGT1A3, UGT1A6 and UGT1A9. UGT1A3 and UGT1A6 have very low intrinsic clearances for SN-38 glucuronidation compared to UGT1A1 and UGT1A9 [42,43]. Both UGT1A1 and UGT1A9 are high affinity enzymes but, whereas UGT1A1 is a high velocity enzymatic reactor for SN-38, the maximum velocity of UGT1A9 for this reaction is approximately 10-fold lower compared to UGT1A1 [42,43]. The intrinsic clearance of UGT1A1 has been reported as being seven-fold higher than that of UGT1A9 in one study [42], whereas another study found that both enzymes had similar catalytic efficiencies [43]. Experiments in our laboratory are in agreement with the first study because we measured an intrinsic clearance for UGT1A1 that was eight-fold higher than that of UGT1A9 [44]. Additional evidence for the main role of UGT1A1 in the glucuronidation of SN-38 is provided by the high association found between the *UGT1A1*28* polymorphism and SN-38 glucuronidation in hepatocytes in the present study, a correlation which is in agreement with numerous reports describing both in-vitro and clinical studies [22,35,45–47]. We believe that UGT1A1, although not 100% specific for SN-38, is one of the best UGT1A1 probes currently available. Moreover, due to the widespread use of complementary medicine products with potentially enzyme-inducing properties in cancer patients [48], we preferred to use SN-38 (the active metabolite of the important anticancer agent irinotecan) to gain insights on the impact of UGT1A1 genetic variation on SN-38 inactivation by glucuronidation under an induced state. Nevertheless because UGT1A9 contributes to some extent to the glucuronidation of SN-38 and phenobarbital induces UGT1A9 [13], it is possible that induction of SN-38 glucuronidation in our system might be a reflection of increased activity of both UGT1A1 and UGT1A9. This is a limitation of our study that could be addressed in future studies by measurement of UGT1A1 and UGT1A9 mRNA levels.

Additional studies are needed to discover the genetic determinants of *UGT1A1* inducibility. Future experiments could be designed to investigate whether polymorphisms in the transcription factors in the PBREM cluster regulating UGT1A1 expression (CAR, PXR, AhR) or in the membrane transporters controlling the efflux of SN-38G and phenobarbital into the media contribute to the variability in inducibility. We studied the classical UGT1A1 inducer, phenobarbital, and future investigations could address whether *UGT1A1* variants affect UGT1A1 inducibility by using activators of other nuclear receptors such as PXR and AhR. Elucidation of the genetic determinants of *UGT1A1* inducibility will help in the treatment of unconjugated hyperbilirubinemia and other diseases for which UGT1A1 substrates are prescribed, and might explain some of the inter-individual differences in drug toxicity and response.

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# Pharmacogenetics of irinotecan: A promoter polymorphism of *UGT1A1* gene and severe adverse reactions to irinotecan

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**Summary**

This review focuses on a pharmacogenetic association between genetic polymorphism of *UGT1A1* gene and severe adverse reactions to irinotecan. Although many studies used pharmacokinetic parameters as surrogate measures for predicting clinical outcomes of irinotecan chemotherapy, they have not produced consistent evidence. On the other hand, genotyping results of *UGT1A1* gene appear to predict severe adverse reactions more straightforward than the pharmacokinetic parameters or the phenotypes of the enzymatic activity. A case-control study of Japanese cancer patients revealed that those with the variant *UGT1A1* alleles were at significantly higher risk of severe adverse reactions to irinotecan, suggesting that the genotyping strategy would be clinically useful. Nevertheless, clinical importance of the pharmacogenetic testing should differ for different patient groups and for different clinical situations. We need to keep this issue in mind in applying the pharmacogenetic evidence in clinical practice.

## Introduction

Patients respond differently to the same medications and a large interindividual variation in drug-related adverse reactions is a major concern in cancer chemotherapy. Drug response is generally associated with systemic exposure to a parent drug or its active metabolite. When clearance of a drug is decreased, residual active compounds can remain in the body longer and may enhance pharmacological effects of the drug. Drug clearance is generally associated with absorption, distribution, metabolism and excretion of a drug, and the drug's metabolism is most essential frequently. Numerous factors are related to a capacity of drug metabolism; for example, age, organ functions, concomitant diseases, food, smoking, environmental chemicals, and drug interactions. Some of these factors inhibit and weaken activity of drug metabolizing effects of the genetic polymorphism are often limited within insignificant pharmacokinetic alternations or have no clinical consequences, if any.

Flavopiridol is the first cyclin-dependent kinase inhibitor to enter clinical trials. The drug is conjugated and detoxified by UDP-glucuronosyltransferase (UGT, EC 2.4.1.17) to yield its $\beta$-glucuronides [1]. UGT1A9 and UGT1A1 are primarily involved in glucuronidation of flavopiridol in humans [2]. Based on a pharmacogenetic study, promoter genotypes of *UGT1A1* gene appeared to be clinically insignificant for pharmacokinetic and pharmacodynamic variations of flavopiridol [3]. In the study, *UGT1A1* gene was genotyped for the promoter polymorphism in 49 patients who were enrolled in a phase I study of the drug. The genotyping results revealed 5 (10%) homozygous and 11 (22%) heterozy-

between the genotypes and the pharmacokinetic parameters, nor the occurrence and severity of diarrhea. In this case, because multiple UGT isoforms are involved in glucuronidation of flavopiridol, the role of UGT1A1 enzyme might be harbored. In fact, based on the *in vitro* study, UGT1A9 plays a more important role in the metabolism than UGT1A1 [2]. Second, despite of the potentially important role of UGT1A1 itself, functional effects of the variant allele might be primarily powerless to affect the pharmacokinetics and pharmacodynamics of the drug. Finally, the genetic effects on UGT1A1 activity might be compensated by acquired non-genetic factors in the studied population.

At present, a few cases have been known that pharmacogenetic testing may be clinically useful to predict clinical outcomes in cancer chemotherapy; thiopurine *S*-methyltransferase, dihydropyrimidine dehydrogenase, and UGT1A1. This review focuses on pharmacogenetic associations between the polymorphisms of *UGT1A1* gene and severe adverse reactions to irinotecan.

## Pharmacokinetics/pharmacodynamics of irinotecan and polymorphisms of *UGT1A1* gene

Irinotecan (CPT-11) is a camptothecin analogue with strong antitumor activity through an inhibition of topoisomerase I. Irinotecan is now widely used, especially for colorectal and lung cancers, but the drug causes unpredictably severe, occasionally fatal, adverse reactions consisting of leukopenia or diarrhea. Irinotecan is a prodrug that is hydrolyzed by carboxylesterase *in vivo* to form an active metabolite SN-38. The SN-38 is subsequently conjugated and detoxified by UGT to yield its $\beta$-glucuronide (SN-38G). Based on the results of *in vitro* experiments, UGT1A1 isoform is responsible for glucuronidation of SN-38 [6, 7]. The glucuronide is excreted in the small intestine *via* bile, where bacterial glucuronidase cleaves the glucuronide into the corresponding SN-38 and glucuronic acid. Regenerated SN-38 was subsequently reabsorbed from the intestine into enterohepatic circulation. Remarkable interindividual variations in the pharmacokinetics have been reported [8–15], which would be due to extensive involvement of multiple enzyme systems (e.g., UGTs, CYP3A4), the protein bindings, and the elimination *via* P-glycoprotein and other transporters [8].

The large interindividual variation in the pharmacokinetic/pharmacodynamic analyses were not consistent, and occurrence of the adverse reactions could not be well explained by use of the pharmacokinetic parameters as surrogates. Complexity of the metabolism and disposition of irinotecan may constitute an obstacle to develop obvious pharmacokinetic/pharmacodynamic relationships, suggesting that the traditional approach targeting plasma exposures of the drug is not likely to succeed to optimize the chemotherapy. Therefore, pharmacogenetic analysis has come to researchers as a promising approach, substituting for the traditional pharmacokinetic methods, to predict severe adverse reactions to irinotecan. Sensitivity to irinotecan would depend on an ability of SN-38 glucuronidation, which is related to an inherited polymorphic activity of UGT1A1 enzyme. Increased exposure to unconjugated SN-38 would result in higher risk of severe adverse reactions to irinotecan in patients who carry variant *UGT1A1* alleles.

UGT is microsomal enzyme that glucuronidates numerous endogenous and exogenous substrates. There are two UGT enzymes in humans, UGT1A and UGT2B. The UGT1A family consists of one gene that locates on chromosome 2q37 along with multiple promoters and the first exons. The gene codes for nine functional proteins, UGT1A1, UGT1A3 to UGT1A10, and four pseudo-genes, UGT1A2p, UGT1A11p, UGT1A12p and UGT1A13p [16]. Each first exon is spliced to the mutual exon 2 and, thus, the substrate specificity of the enzyme depends on the first exon. UGT1A1 gene is composed of a promoter and the first exon closest to the exons 2 through 5 [17, 18]. Primarily, UGT1A1 enzyme is responsible for conjugating bilirubin in the body and it glucuronidates drugs (e.g., ethinylestradiol), xenobiotic compounds (e.g., phenols, anthraquinones and flavones) and endogenous steroids [19]. As of December 2004, a total of 64 genetic variations of the gene have been reported in the promoter region and exons http://som.flinders.edu.au/FUSA/ClinPharm/UGT/allele_table.html. These genetic variations decrease the enzymatic activity and cause constitutional unconjugated jaundice, known as Crigler-Najjar syndromes I & II and Gilbert's syndrome [20]. Crigler-Najjar syndromes are rare genetic traits characterized by absent or very low UGT1A1 activity. On the other hand, hyperbilirubinemia in a patient with Gilbert's syndrome is milder than that observed in Crigler-Najjar syndromes, and 3 to 10% of the general population is estimated to

(+211 from the initial site of the transcription, G to A) at codon 71 in exon 1 that changes glycine to arginine (G71R, *UGT1A1*6*); a transversion (+686, C to A) at codon 229 in exon 1 that alters proline to glutamine (P229Q, *UGT1A1*27*); a transversion (+1099, C to G) at codon 367 in exon 4 that converts arginine to glycine (R367G, *UGT1A1*29*); and a transversion (+1456, T to G) at codon 486 in exon 5 that transforms tyrosine into aspartic acid (Y486D, *UGT1A1*7*). Decrease in the UGT activity by these genetic variants that are associated with Gilbert's syndrome is relatively small, and individuals carrying the variants do not always show hyperbilirubinemia [4, 5]. However, because of narrow therapeutic windows of anticancer drugs, even a small decrease in the enzyme function may be critical in cancer chemotherapy. Furthermore, cancer patients having these genotypes are easily overlooked by routine physical examinations or laboratory analyses, and they would be candidates for irinotecan-containing chemotherapy.

Recent pharmacogenetic studies have demonstrated that genotyping of *UGT1A1* gene explains some of the pharmacodynamic variations of irinotecan [21–26]. A case-control study of 118 Japanese cancer patients who had been treated with irinotecan revealed that those with variant *UGT1A1* alleles were at significantly higher risk of developing severe adverse reactions to the drug [21]. Twenty-six of the 118 patients experienced severe adverse reactions consisting of grade 4 leukopenia ($\leq$ 900/$\mu$l) and/or grade 3 (watery for 5 days or more) or grade 4 (hemorrhagic or dehydration) diarrhea, and the remaining 92 patients did not [27]. Of the 26 patients with severe adverse reactions, the genotypes of *UGT1A1*28* were homozygous in 4 (15%) and heterozygous in 8 (31%), while 3 (3%) homozygous and 10 (11%) heterozygous were found among the 92 patients without severe adverse reactions. Multivariate analysis suggested that the genotype either heterozygous or homozygous for *UGT1A1*28* would be a significant risk factor for severe adverse reactions (odds ratio, 7.23; 95% confidence interval, 2.52–22.3). In other words, the patients either heterozygous or homozygous for *UGT1A1*28* were 7 times as likely to encounter severe adverse reactions in the studied population. All 3 patients (3%) who were heterozygous for *UGT1A1*27* encountered the severe adverse reactions. No statistical association was observed between *UGT1A1*6* and the occurrence of severe adverse reactions. None had *UGT1A1*29* or *UGT1A1*7* in under the supervision of specialists who were thoroughly experienced in cancer chemotherapy [29]. Each patient had to be registered and be judged appropriate for administration of the drug prior to the treatment, and their clinical outcomes, including adverse reactions, had to be reported during the surveillance. While an association of UGT activity with severe adverse reactions to irinotecan had been implied by the previous case report [30], this research was the first controlled study to verify the putative association of *UGT1A1* genetic polymorphisms with clinical outcomes directly. A style of the study would be representative to identify nested populations who are likely to have response or therapeutic failure [31].

Associations between *UGT1A1*28* and pharmacokinetic parameters of irinotecan also have been investigated [32–35]. The first study showed that ratios of SN-38 to SN-38G concentrations following irinotecan administration were markedly increased in a patient homozygous for *UGT1A1*28*, where the ratios were used as a surrogate for UGT activity [32]. An additional study using pooled pharmacokinetic data of irinotecan explored polymorphic frequency distribution of AUC ratios of SN-38 to SN-38G ($AUC_{SN-38}/AUC_{SN-38G}$) [33]. The data from 100 Japanese patients, including 14 who were genotyped for *UGT1A1* gene, was analyzed. The frequency distribution of $AUC_{SN-38}/AUC_{SN-38G}$ was skewed to the right without apparent bimodality. The skewed distribution suggests potential existence of a subpopulation due to the continuous measure rather than a discrete distribution. The subjects carrying *UGT1A1*28* showed the values that were contained in the stretched tail on the right side of the distribution, suggesting a potential pharmacogenetic/pharmacokinetic relationship. However, the patients could not be segregated into discrete subgroups by UGT activity. This finding doubt an idea that phenotypes of UGT1A1 activity would be clinically useful parameters for identifying patients at higher risk of severe adverse reactions to irinotecan.

We are of the opinion that *UGT1A1* genotype would be more straightforward predictor of severe adverse reactions to irinotecan than the pharmacokinetic parameters or the phenotype of enzymatic activity. As discussed above, many studies analyzed the pharmacokinetic parameters as the surrogate measures and have not produced reliable evidence. When the genotyping results do not reflect plasma exposures to the active compounds appropriately, it is not surprising that relationships observed between

the pharmacokinetic parameters were not significantly related to any of the variant genotypes investigated. As regards *UGT1A1*28*, the ability of SN-38 glucuronidation was impaired in two homozygous patients, albeit the difference was not statistically significant. In contrast, one of the two homozygous patients was the only one who experienced grade 4 diarrhea in the entire patients. This finding supports that the genotyping would be more useful tool to predict severe adverse reactions than the pharmacokinetic parameters. Based on the pharmacology of irinotecan, it might be expected that baseline bilirubin levels could be a handy predictor of clinical outcomes of irinotecan chemotherapy. In fact, patients with overt jaundice are at higher risk of severe adverse reactions and are usually excluded from irinotecan use. However, when the baseline bilirubin levels are within the normal range, while the association with severe adverse reactions has been reported to be statistically significant, the bilirubin levels are not strong predictors enough to be used as a tool in clinical practice [21, 36].

### Additional factors affecting a clinical importance of *UGT1A1*28* to predict severe adverse reactions to irinotecan

It has been known that there are great interethnic differences in the distributions of *UGT1A1* genetic polymorphisms [37–39]. The allele frequency of *UGT1A1*28* among Caucasians is higher (0.3 to 0.4) than that among Japanese (about 0.15). On the contrary, variant alleles in the coding regions were found only in Japanese and Asians; the frequencies are 0.11 to 0.23 for *UGT1A1*6* and 0.01 to 0.03 for *UGT1A1*27*. In other words, most Japanese and Asian patients with Gilbert's syndrome have the variant sequence in the coding regions, while those among Caucasians have it in the promoter. The same variants might be less predictive surrogates of drug effects in other populations in which other variants are more or less frequent. Nevertheless, *UGT1A1*28* as a hereditary predictor of severe adverse reactions has been validated in multiple races beyond the underlying genetic differences [22–25].

Besides *UGT1A1*28*, other *UGT1A1* genotypes may modify clinical significance of *UGT1A1*28*. A variant *UGT1A1*60*, which exists in the phenobarbital-multivariate analysis when controlling for *UGT1A1*28*, due to the highly significant linkage disequilibrium between the two alleles. As regards *UGT1A1*6*, neither the pharmacokinetic parameters nor occurrence of severe adverse reactions were related to this valiant allele at least as a single genetic marker [21, 35]. One more polymorphism in the promoter (-3156G>A) has recently been reported as potentially better predictor than *UGT1A1*28* [23]. Functional effect of the variant on UGT enzyme activity need to be verified and the clinical significance requires further evaluations in other patient populations.

Other drug metabolizing enzymes/transporters together with genetic polymorphisms may affect the clinical role of *UGT1A1*28*. In addition to UGT1A1, UGT1A9 and UGT1A7 are responsible for glucuronidation of SN-38, and ATP-binding cassette (ABC) gene products, including ABCB1 (P-glycoprotein), ABCC2 (multidrug resistance-associated protein 2), and ABCG2 (breast cancer-resistance protein) are involved in an elimination pathways of irinotecan [8, 41]. However, the additional analyses in the Japanese study found no significant associations between the variants of *UGT1A7* or *ABCC2* genes and severe adverse reactions [42, 43]. Recently, *UGT1A9* polymorphisms (*UGT1A9*5* and *UGT1A9*3*) have been studied in 94 Caucasian patients treated with irinotecan [44]. The study showed that only one patient had *UGT1A9*3* heterogeneously and did not experience diarrhea or neutropenia. The authors concluded that the *UGT1A9* variants were unlikely to be clinically significant. The *ABCB1* 1236C>T variant, which was related to pharmacokinetic alternations of irinotecan in the comprehensive study, should be studied further to confirm its clinical significance [34]. No significant pharmacokinetic changes were found in relation to *ABCG2* 421C>A genotype in 84 Caucasian patients who were treated with irinotecan [45].

Finally, different diets and lifestyles may affect the protein functions. The variation in the enzyme function can be caused by stimulation or inhibition of enzyme, which is particularly important in cancer patients who are receiving multiple drugs against palliation. Patients with malignant glioma showed lower ratio of $AUC_{SN-38}/AUC_{SN-38G}$, which may be due to the induction of UGT1A enzyme by chronic uses of anticonvulsants and steroids [46]. On the other hand, patients who concurrently used St. John's Wort

*Table 1.* Associations between *UGT1A1* genotypes and severe adverse reactions to irinotecan

| | Severe adverse reactions[a] | | |
|---|---|---|---|
| *UGT1A1*28* | Experienced | Not experienced | Totals |
| Yes | 12 | 13 | 25 |
| No | 14 | 79 | 93 |
| Totals | 26 | 92 | 118 |

[a] Leukopenia (grade 4) and/or diarrhea (grade 3 or worse) defined by the Japan Society for Cancer Therapy criteria [27].

by midazolam clearance as a probe, is significantly associated with irinotecan pharmacokinetics [48].

## Conclusion

In the Japanese case-control study, genotyping result of presence of *UGT1A1*28* is 46% (12/26) sensitive and 86% (72/92) specific to severe adverse reactions to irinotecan, resulting in a positive likelihood ratio of 3.3 (Table 1) [21]. Thus, the positive predictive value of the genotyping test appears to be 48% (12/25), assuming that the table approximates the true prevalence of the event. By determining which patients are at higher risk for severe adverse reactions based on the their genetic status, we would be able to reduce dosage or switch irinotecan to others to avoid unnecessary adverse events [31, 49]. Nevertheless, clinical importance of pharmacogenetic testing should differ for different patient groups and for different clinical situations (e.g., diseases, drug interactions, and dose intensity). We need to keep this issue in mind in applying the pharmacogenetic evidence in clinical practice.

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